Plant production and immunogenic characterisation of Human papillomavirus chimaeric vaccines

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Plagiarism declaration

I, Catherine Pineo, declare that this thesis / dissertation, submitted for the degree of Master of Science at the University of Cape Town, under the co-supervision of Prof. Edward Rybicki and Dr Inga Hitzeroth, is my own work.

Signature:

Date:

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"If I have seen further than others, it is by standing on the shoulders of giants."
~ Isaac Newton

Abstract

Cervical cancer is primarily caused by infection with Human papillomavirus (HPV) and is a global concern, particularly in developing countries which contain ~80% of the cervical cancer burden. Current HPV L1 major capsid protein virus-like particle (VLP)-based vaccines are effective in the type-specific prevention of infection and associated disease. However, the high cost of the vaccines has limited their widespread application, and cytological screening programmes are still required to detect malignant lesions associated with the non-vaccine types, particularly in HIV-infected populations. Furthermore, the vaccines lack therapeutic efficacy in the treatment of established HPV infections and disease. As a result, there is an urgent demand for cheaper second-generation HPV vaccines, preferably novel vaccines which are therapeutic, and broadly protect against several HPV types.

The objective of this study was to express novel HPV-16 L1-based chimaeras in plants using transient and transgenic tobacco expression systems, and investigate the immunogenicity of three L1 chimaeric candidate vaccines containing sequences of the L2 minor capsid protein. Eight L1 chimaera candidate vaccines were designed with cross-protective L2 epitopes and therapeutic E7 CTL epitope sequences replacing regions of the L1 C-terminal. The L1/L2 chimaeras contained epitope sequences derived from HPV-16 L2 aa 108-120, 56-81 or 17-36 and BPV-1 L2 1-88 substituted into the helix 4 (h4) region of L1 at aa 414. The L1/E7 chimaeras contained the HPV-16 E7 CTL epitopes aa 49-57 and aa 86-93 (used in HPV-related cancer immunotherapy studies in mice and humans respectively) substituted in a similar L1 region at aa 417. Furthermore, two L1/L2/E7 chimaeras were analysed and contained the L2 aa 108-120 epitope at aa 414 and an additional E7 epitope in the coil between h4 and the β-J region at aa 433/434.

All chimaeras were expressed in plants using an *Agrobacterium*-mediated transient system, and maximum yields were obtained by targeting the

expressed protein to the chloroplast. The L1/E7 and L1/L2/E7 chimaeras were poorly expressed in plants, with yields of 30 – 80 mg/kg plant tissue, and require further optimisation. Transient and transgenic plant expression studies suggest the insertion of two epitopes in the L1/L2/E7 chimaeras negatively affects chimaera expression.

The L1/L2 chimaera with the BPV L2 aa 1-88 epitope replacing the C-terminal of L1 was not well-expressed and degradation was detected, suggesting expression of this chimaera is not viable in plants. Three L1/L2 chimaeras containing the L2 aa 108-120, 56-81 and 17-36 epitopes were highly-expressed with yields of ~1200 mg/kg plant tissue, and chimaeras assembled differently, indicating that the length of the L2 epitope affects VLP assembly. The L1/L2 chimaera containing L2 aa 108-120 epitope was the most successful candidate vaccine. It assembled into small VLPs, elicited anti-L1 and L2 responses and antisera neutralised pseudovirions from homologous HPV-16 and heterologous HPV-52, which are detected in 50-60% of cervical cancers and are highly prevalent in cervical lesions in South Africa. The other L1/L2 chimaeras containing longer L2 sequence replacements predominantly assembled into capsomeres and aggregates and elicited lower humoral immune responses.

In summary, the expression of HPV-16 chimaera candidate vaccines in plants was investigated and optimized using several methods. The immunogenicity and the potential of HPV-16 L1/L2 chimaeras to display the L2 epitopes was analysed and the results demonstrated the importance of VLP assembly in the immunogenicity of the candidate vaccines.

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Chapter 1: Literature Review

1.1 Introduction

Human papillomaviruses (HPVs) are small, double-stranded, non-enveloped DNA viruses that infect human squamous and cutaneous epithelial cells (zur Hausen, 2000). The virus genome is approximately 8 kb in size (Seedorf *et al*., 1985) and encodes six early genes (E1, E2, E4, E5, E6, and E7) and two late genes (L1 and L2). The structure and function of the encoded proteins is shown in Figure 1 (Lin *et al*., 2010).

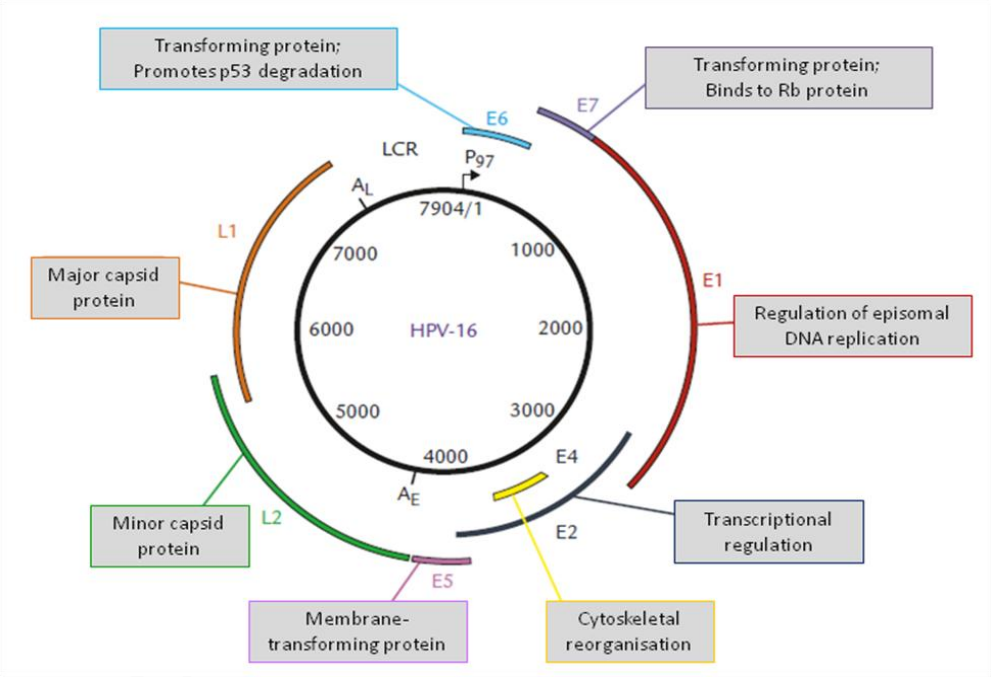

Figure 1: HPV-16 genome and encoded protein function. The transcriptional promoter is designated P97. AE and AL are the early and late polyadenylation sites, respectively. The viral long control region (LCR) contains transcriptional and replication regulatory elements. The HPV-16 genome contains six early genes, E1, E2, E4, E5, E6, E7, and two late genes, L1 and L2 (modified from Lin *et al*., 2010).

The late genes encode the structural capsid proteins: the major capsid protein (L1) and the minor capsid protein (L2). The capsid shell comprises of 360 copies of L1 assembled into 72 pentamers (capsomeres) with 36-72 copies of L2 (Buck *et al*., 2008) arranged in a T=7 icosahedral lattice (Trus *et al*., 1997; Buck *et al*., 2005). L1 spontaneously assembles into empty virus-like particles

(VLPs), in the presence or absence of L2 (Kirnbauer *et al*., 1992). Although the VLPs lack the virus genome DNA, their morphological and immunological characteristics are very similar to those of native papillomaviruses (Kirnbauer *et al*., 1992, 1993; Rose *et al*., 1994a; Hagensee *et al*., 1993).

The family *Papillomaviridae* consists of 16 genera, with 5 of these containing HPVs: the Alpha, Beta, Gamma, Mu and Nu papillomaviruses (de Villiers *et al*., 2004). HPVs comprise of more than 120 epitheliotropic genotypes, which are sub-grouped into cutaneous or mucosal types according to their ability to infect the skin or mucosa of the genital or the upper-respiratory tracts. Approximately 40 of these types specifically infect the genital tract (Muñoz *et al*., 2003), and 12 are classified as human carcinogens by the International Agency for Research on Cancer (IARC) (Bouvard *et al*., 2009).

The HPV types that infect the genital mucosa are divided into two groups. The low-risk types, including HPV-6 and 11, cause benign condylomas. The high-risk types, such as HPV-16 and 18, are strongly associated with the development of malignant disease (Muñoz *et al*., 2003). HPV types are classified on the basis of L1 gene sequence homology. With rare exception, most of the known HPV types that infect the genital tract are members of the Alphapapillomavirus (A) genus (de Villiers *et al*., 2004).

1.2 The global burden of cervical cancer

Cervical cancer is the third most common cancer among women worldwide, with 529 000 incident cases and 275 000 attributable deaths in 2008 (Ferlay *et al*., 2010). More than 80% of cases arise in developing countries where health care resources are limited (Parkin and Bray, 2006). The global distribution of cervical cancer is shown in Figure 2, with majority of cases occurring in Africa, South America and South East Asia.

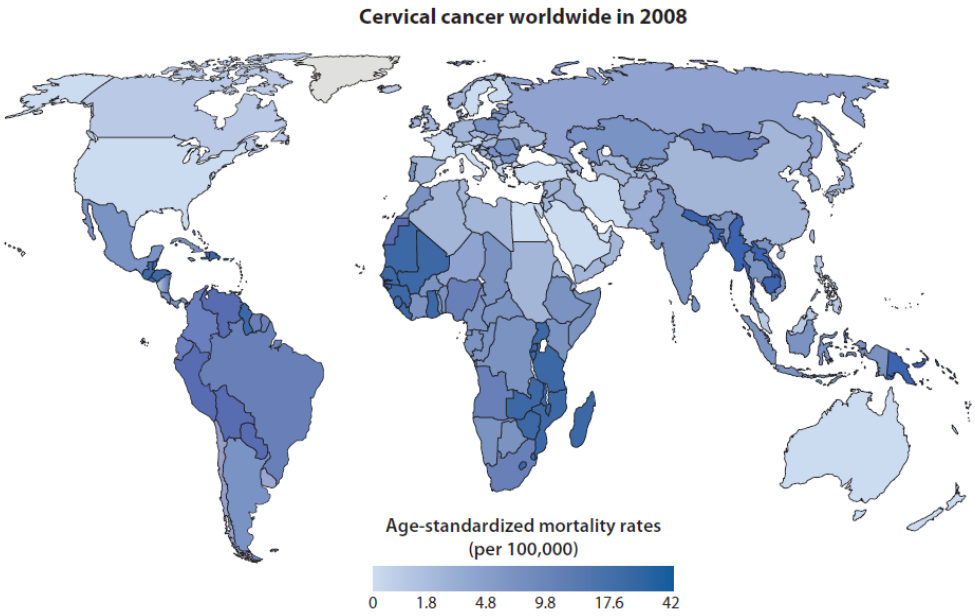

Figure 2: Distribution of cervical cancer deaths worldwide as reported through Globocan (Ferlay *et al*., 2010).

Carcinoma of the cervix is associated with HPV in more than 99% of cases (Walboomers *et al*., 1999), and the causal association between HPV infection and cervical cancer has been well-described (zur Hausen *et al*., 1981). HPV has also been established as a cause of cancer of the penis, vulva, anus, vagina and oropharynx (Münger *et al*., 2004), and therefore HPV vaccine development is a prime priority for preventative cancer research.

The majority of HPV infections, regardless of type, are transient and clear spontaneously, typically within 1 to 2 years (Schiffman *et al*., 2005), although resolution is less frequent and substantially delayed in immuno-compromised subjects (Koshiol *et al*., 2006). Approximately 10% of women fail to clear high-risk HPV-type infections and long-term persistent HPV infection is a prerequisite for the development of cervical cancer (Stanley, 2008).

HPV-16 is the most prevalent genotype among women with invasive cervical cancers, followed by HPV-18, thus these types are the major etiological agents of cervical cancer and account for approximately 70% of diagnosed cervical cancers worldwide (Smith *et al*., 2007a, de Sanjosé *et al*., 2010). Although their hierarchy may differ, the six most common HPV types are

HPV-31, 33, 35, 45, 52 and 58, as consistently reported by the global IARC pooled analysis (>3000 cases, Muñoz *et al*., 2004), meta-analyses (>10,000 cases, Clifford *et al*., 2003; >14,500 cases, Smith *et al*., 2007a; >30,800 cases, Li *et al*., 2011) and a recent cross-sectional world-wide study (>10,500 cases, de Sanjosé *et al*., 2010).

HPV is highly prevalent in South Africa and is detected in 20-26% of women (Williamson *et al*., 2002; Mqoqi *et al*., 2004; Marais *et al*., 2008a). Cervical cancer is the second most common cancer among South African women and the most prevalent in black women (Mqoqi *et al*., 2004), with 6800 women diagnosed each year and 3700 annual deaths (Sinanovic *et al*., 2009).

These statistics highlight the significant burden of HPV infection and its associated disease, particularly in developing countries with high morbidity and mortality rates (Cutts *et al*., 2007). Screening programmes have been ineffective in reducing the incidence of cervical cancer in developed countries (Lowy *et al*., 2008), and given that prophylactic vaccines are an effective strategy to prevent HPV infections and associated disease (WHO, 2005), there is an urgent demand for HPV vaccines, both globally and locally in South Africa.

1.3 Prophylactic HPV vaccines

The L1 major capsid protein is the antigen of choice in the development of prophylactic (preventative) vaccines. Expression of L1 results in the spontaneous self-assembly of virus-like particles (VLPs) which are similar both morphologically and immunogenically to native virions (Zhou *et al*., 1991a; Kirnbauer *et al*., 1992; Rose *et al*., 1994a) and thus retain immunogenic epitopes found on authentic virions without containing the infectious genome.

Immunization with VLPs induces the production of neutralising antibodies (NAb) which protect animals from subsequent challenge with infectious virus (Breitburd *et al*., 1995; Jansen *et al*., 1995; Suzich *et al*., 1995; Christensen *et*

al., 1996; Kirnbauer *et al*., 1996) and are predominantly directed against type-specific conformational epitopes (Christensen and Kreider, 1990; Kirnbauer *et al*., 1992; Roden *et al*., 1994; Rose *et al*., 1994b).

The well-established link between HPV infection and cervical cancer (Wallboomers *et al*., 1999), as well as the high prevalence of HPV infection (Smith *et al*., 2007a), has lead to the development of prophylactic vaccines directed against the most relevant high-risk oncogenic HPV types (Christensen, 2005). Early adolescent females are the primary target of preventative HPV vaccines, as up to 48% of women will have evidence of cervical human papillomavirus (HPV) infection within 3 years after initiating sexual activity (Collins *et al*., 2002).

Two HPV L1 VLP-based prophylactic vaccines have been licenced and are commercially available (reviewed by Lin *et al*., 2010). Gardasil® (Merck & Co., Inc.) is a quadrivalent HPV-6/11/16/18 vaccine, containing L1 VLPs of low-risk types 6 and 11, and the high-risk oncogenic types 16 and 18. The VLPs are produced in a *Saccharomyces cerevisiae* expression system and are adsorbed onto amorphous aluminum hydroxyphosphate sulfate (AAHS) adjuvant. Cervarix™ (GalaxoSmithKline Biologicals) is a bivalent HPV-16/18 vaccine containing L1 VLPs from HPV types 16 and 18, produced in recombinant baculovirus insect cells (*Trichoplusia ni*) and adjuvanted with 3-O-desacyl-4´-monophosphoryl lipid A and aluminium hydroxide (AS04). The vaccines are expensive (US $300-360 for a 3 dosage regiment), administered by intramuscular injection and require refrigeration (Schiller *et al*., 2008).

Vaccine safety, immunogenicity and efficacy has been demonstrated for both Gardasil® and Cervarix™ in Phase II and III clinical trials (Harper *et al*., 2006; Villa *et al*., 2006; FUTURE II Study Group, 2007a; Garland *et al*., 2007; Paavonen *et al*., 2007), summarized and reviewed by Schiller *et al*. (2008). Prophylactic vaccine efficacy was defined by the absence of HPV vaccine-type infections and/or associated disease endpoints, particularly high-grade Cervical Intraepithelial Neoplasia (CIN2+) for both vaccines, as well as Vulvar Intraepithelial Neoplasia (VIN) or Vaginal Intraepithelial

Neoplasia (VaIN) and genital warts, for the Gardasil vaccine. The study of primary efficacy was done using women who were both DNA and seronegative for HPV vaccine types prior to immunisation.

Results show vaccines are well-tolerated, highly immunogenic and prevent vaccine-type infections and HPV-related CIN2+ and cervical adenocarcinoma in situ (AIS) in young women (Harper *et al*., 2004, 2006; Villa *et al*., 2005, 2006; Ault *et al*., 2007a; FUTURE II Study Group, 2007a, 2007b; Garland *et al*., 2007; Joura *et al*., 2007, 2008; Paavonen *et al*., 2007, 2009). Furthermore, women previously exposed to one or more HPV vaccine types prior to Gardasil immunisation did not experience reinfection or reactivation of vaccine-type associated disease (Olsson *et al*., 2009). The sustained safety and protective efficacy for vaccine HPV type infections and associated disease has been demonstrated up to 6.4 years for Cervarix (Harper *et al*., 2006; David *et al*., 2009) and 8.5 years for Gardasil (Villa *et al*., 2006; Rowhani-Rahbar *et al*., 2009) following vaccination.

Low levels of cross-neutralising antibodies against closely-related HPV types are elicited by the L1 VLP vaccines (Brown *et al*., 2009; Ault, 2007b; Harper *et al*., 2004, 2006; Paavonen *et al*., 2007, 2009; Bonanni *et al*., 2009). In particular, cross-reactivity has been observed between HPV-16, 31, 33, and 58 and between HPV-18 and 45, however the efficacy and duration of the cross-neutralising antibody responses are currently unknown (Kwak *et al*., 2011). Cervarix has also shown cross-protection against incident infection with HPV-52 (Harper *et al*., 2004), although efficacy against HPV-52 may not be sustained over time (Harper *et al*., 2006), and recently cross-protection against individual HPV-31, 33 and 45 type-associated CIN2+ lesions has been reported by Paavonen *et al*. (2009).

1.4 Limitations of current HPV vaccines

It should be stated that the multi-national HPV phase II and III trials were not inclusive of all geographical regions, most noticeably Africa, and long-term monitoring is still required to determine safety, sustained immune responses

and vaccine disease efficacy (Dillner *et al*., 2007; Schiller *et al*., 2008). Also, the initial trials were not representative of all gender and age populations, although other small-scale clinical trials analysing efficacy in males and mature women have recently been published (Reisinger *et al*., 2007; Block *et al*., 2006; Pederson *et al*., 2007; Muñoz *et al*., 2009; Olsson *et al*., 2009; Schwarz *et al*., 2007; Castellsagué *et al*., 2009; Bonanni *et al*., 2009).

Despite the high efficacy of both vaccines, there are several important concerns including: (a) the vaccine type-restricted prophylactic efficacy, which limits protection in different populations given the geographical and regional variation in HPV type prevalence and distribution, (b) the lack of therapeutic efficacy and (c) the high cost of vaccines.

1.4.1 Type-specificity of L1-based HPV vaccines

Over a dozen high risk HPV genotypes are commonly associated with cervical cancers worldwide (Muñoz *et al*., 2004) and thus the development of vaccines protecting against multiple HPV types is a public health priority. Although cross-protective responses have been reported for the vaccines, *in vitro* neutralisation studies indicate that cross-neutralising antibody titres are low (Smith *et al*., 2007b). Furthermore, the limited cross-protective efficacy of vaccines is evident in analyses including lesions associated with non-vaccine types, particularly in intention-to-treat (ITT) groups which incorporates women with prevalent HPV infections and thus offer the best indication of vaccine protection in a partially-infected general population (Schiller *et al*., 2008).

Data from the Gardasil FUTURE I and II trial indicated vaccine efficacy in the prevention of external genital lesions, CIN2/3 and AIS were 34%, 20% and 17% respectively in ITT groups (Schiller *et al*., 2008). Paavonen *et al*. (2009) reported Cervarix efficacy was only 37-53% against CIN2+ lesions associated with non-vaccine oncogenic HPV types. Furthermore, cross-protection elicited by Gardasil was not effective against high-grade cervical lesions (CIN2+) associated with non-vaccine HPV types in the ITT group (Wheeler et al., 2009), in contrast to the HPV-naive group (Brown *et al*., 2009).

An important consideration in HPV vaccine development, particularly in an African context, is the geographical and regional differences in HPV genotype prevalence and associated disease (de Sanjosé *et al*., 2010; Castellsagué *et al*., 2006; Smith *et al*., 2007a) and the prevalence of HPV in Human immunodeficiency virus (HIV)-infected populations (Clifford *et al*., 2006).

Although HPV prevalence is highly variable among different populations, the most common high-risk HPV types in asymptotic women are HPV-16, 18, 52, 31, 58, 39, 56 and 51 (Bruni *et al*., 2010), suggesting prophylactic vaccines should prioritise protection against these types. Sub-Saharan Africa has the highest HPV prevalence in the world, with a prevalence of 24% in comparison to the global estimate of 11% (Bruni *et al*., 2010). HPV-positive women in Africa are significantly less likely to be infected with HPV-16 and more likely to be infected with other HPV types than European women (Clifford *et al*., 2005), and contain a greater burden of non-vaccine type HPV infections (Bosch *et al*., 2009), indicating broadly protective vaccines are particularly essential in Africa.

A recent study on the geographical HPV genotype distribution in cervical cancers was published by de Sanjosé *et al*. (2010). The global HPV type prevalence and African estimates are compared in Figure 3. The cumulative prevalence of the vaccine types HPV-16 and 18 in African cervical adenocarcinomas is 71% (similar to the global estimate). However, Africa has the lowest proportion of cervical cancer attributable to HPV-16, and the HPV types 18, 45, 35 and 51 are all more prevalent then global estimates. Furthermore, multiple HPV infections are detected in 26-48% of HPV-infected women in South Africa (Marais *et al*., 2008; Said *et al*., 2009) and 19% of cervical abnormities in Africa are associated with multiple HPV infections, which is more than double the global estimates of 9% (de Sanjosé *et al*., 2010). As a result, broader protection against non-vaccine HPV types is needed.

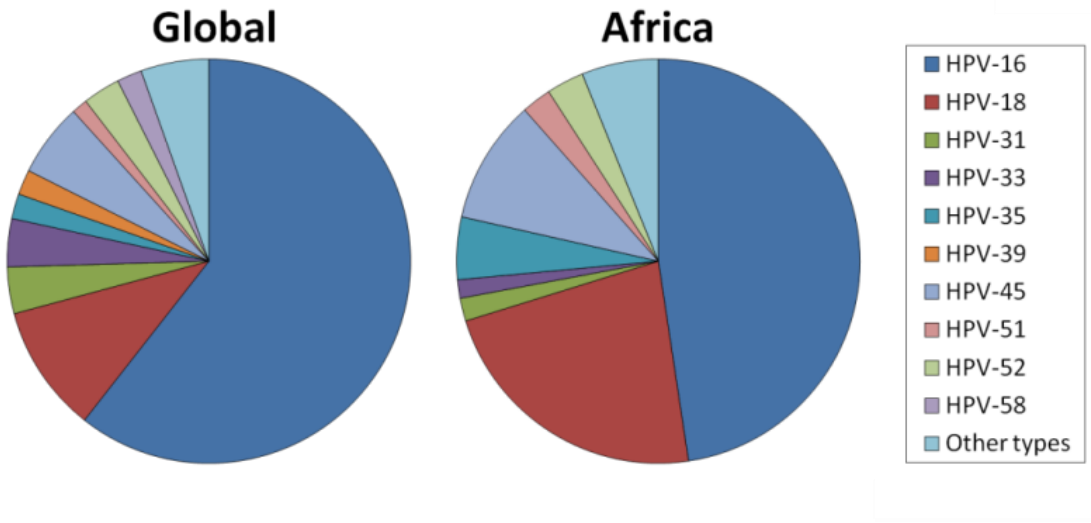

Figure 3: Global and African prevalence of HPV types in invasive cervical cancer globally. The data was taken from de Sanjosé *et al*. (2010) and modified.

The high prevalence of HPV-35 in sub-Saharan Africa has been reported both for asymptotic women (Clifford *et al*., 2005) and in women with high-grade cervical cancer; specifically in South Africa (Allan *et al*., 2008; Said *et al*., 2009), Nigeria (Clifford *et al*., 2005), Kenya (De Vuyst *et al*., 2003) and Mozambique (Castellsagué *et al*., 2001). Other non-vaccine types which were highly prevalent in cervical cancer include HPV-31, 33, 45, 52, 53 and 58 (Kay *et al*., 2003; Naucler *et al*., 2007; Allan *et al*., 2008; Marais *et al*., 2008a; Banura *et al*., 2008; Blossom *et al*., 2007; De Vuyst *et al*., 2003; Said *et al*., 2009; Xi *et al*., 2003; Louie *et al*., 2009).

A recent South African study demonstrated HPV-16, 18 and 35 prevalence was associated with increasing cervical disease severity (Allen *et al*., 2008). Importantly, HPV-16 only predominated in women with high-grade squamous intraepithelial lesions (HSILs), in whom HPV-35 was equally prevalent (18.9%). Furthermore, HPV-52 was the most prevalent in low-grade squamous intraepithelial lesions (LSILs, 17.5%) and highly prevalent with HPV-31 in HSIL (11.3%), thus the non-vaccine types HPV-31, 35 and 52 are of significant concern in South Africa.

Importantly, the efficacy of these vaccines has not been evaluated in immuno-compromised populations (Villa, 2011), particularly in HIV-infected women who have a higher risk of acquiring HPV infections and developing HPV-related diseases (Palefsky, 2006; Clifford *et al*., 2006; Moodley *et al*., 2006; 2009; Baay *et al*., 2004; Adler *et al*., 2008; Louie *et al*., 2009). In particular, African HIV-positive women have the highest prevalence of HPV infection worldwide (Clifford *et al*., 2006) and several African studies have indicated HPV-16 does not predominate to the same extent as seen in HIV-negative women (Moodley *et al*., 2009; Sahasrabuddhe *et al*., 2007; Blossom *et al*., 2007; Singh *et al*., 2009; Banura *et al*., 2008).

In South Africa, Marais *et al*. (2008b) demonstrated HPV prevalence in HIV-positive woman is more than double compared to HIV-negative women (50% and 24% respectively). Although HPV-16 is the most prevalent type in HIV-positive women with cervical cancer, other important types in South Africa include HPV-45 (Marais *et al*., 2008b), HPV-51 and 58 (Moodley *et al*., 2009) and HPV-52, 53 and 35 (Denny *et al*., 2008). As a result, broadly protective vaccines are a priority in these populations.

1.4.2 The lack of therapeutic efficacy

Another concern is the therapeutic treatment of HPV infections in women who have been previously exposed to HPV. Although HPV vaccination has been shown to induce cell-mediated immune responses traditionally involved in the eradication of infections (Giannini *et al*., 2006; Pinto *et al*., 2003; 2005; Emeny *et al*., 2002; Peng *et al*., 1998), neither Gardasil (FUTURE II Study Group, 2007b) nor Cervarix (Hildersheim *et al*., 2007) demonstrated therapeutic efficacy against prevalent HPV infections or lesions, with no effect on the rate of viral clearance or HPV-related disease progression (Schiller *et al*., 2008).

1.4.3 High vaccine costs

HPV vaccines will potentially have the greatest impact in developing countries, which have the highest burden of HPV-related disease as a result of poor health resources (Parkin and Bray, 2006). One of the biggest hurdles for the widespread application of these vaccines is their expense, particularly

as vaccine type-specificity does not eradicate the need for cytological screening programmes to detect cervical cancers caused by the non-vaccine HPV types (Kwak *et al*. 2011). In South Africa, estimates for a cost-effective vaccination and screening programme suggest a reduction of >60% is needed (Sinanovic *et al*. 2009). Therefore there is an urgent demand for cheaper second-generation HPV vaccines.

1.5 Second-generation prophylactic HPV vaccines

The necessity of further HPV vaccine development is called into question with two highly-effective prophylactic vaccines available. However, the cost and limited protection of the vaccines have provided an incentive for the development of improved second-generation HPV vaccines.

1.5.1 Broadening protection using L2 cross-neutralising epitopes

Although the immunogenicity and efficacy of HPV vaccines has been well-demonstrated, the cross-protection against other oncogenic HPV types is partial and limited to closely related genotypes. As a result of the type-restriction of vaccines, it remains important to broaden protection to include the remaining oncogenic HPV types.

The L2 minor capsid protein has emerged as a candidate for the development of prophylactic HPV vaccines (Gambhira *et al*., 2006; Alphs *et al*., 2008; Karanam *et al*., 2009). The N-terminal region of L2 is of particular interest as it contains several epitopes (surface-exposed immunogenic regions displayed in a specific three dimensional conformation) which induce the production of cross-neutralising antibodies against a broad range of papillomavirus types (Roden *et al*., 2000; Kawana *et al*., 1999, 2001, 2003; Embers *et al*., 2004; Pastrana *et al*., 2005; Palmer *et al*., 2006; Gambhira *et al*., 2007a; Kondo *et al*., 2007, 2008; Alphs *et al*., 2008; Schellenbacher *et al*., 2009) and elicits the production of neutralising antibodies which protect *in vivo* (Gaukroger *et al*., 1996; Embers *et al*., 2002).

A structural sequence analysis shows the L2 amino acid (aa) 1-120 region to be highly conserved (Lowe *et al*., 2008). Several areas within this region are broadly neutralising and studies using overlapping L2 peptides have been utilized to map the important cross-neutralising epitopes (Pastrana *et al*., 2005; Schellenbacher *et al*., 2009; Rubio *et al*., 2009; Rubio *et al*., 2011). Three highly-conserved neutralising epitopes within HPV-16 L2 N-terminal have been identified: aa 90-122, 56-81 and 17-38.

(a) L2 epitopes within aa 90-122

The first major region investigated for cross-neutralisation is aa 90-122. The cross-neutralising HPV-16 L2 epitope comprising of aa 108-120 has shown to generate sera which cross-neutralises HPV-6 (Kawana *et al*., 1999) and HPV-52 (Kawana *et al*., 2003). Additionally, fusion of the peptide to keyhole limpit haemocyanin (KLH) induced cross-neutralising antibodies against native HPV-11 virions (Slupetzky *et al*., 2007).

Several L2 peptides overlapping this region were examined, including aa 90-111, 96-115 and 107-122. Only the L2 peptide composed of aa 96-115 cross-neutralised HPV-31 and 58, suggesting the regions unique to this peptide (aa 96-107 and aa 111-115) are essential in the epitope, although mutating aa 101 and 112 had no affect on cross protection (Kondo *et al*., 2007). A similar observation was reported by Embers *et al*. (2002), whereby the overlapping L2 region aa 107-112 of cottontail rabbit papillomavirus (CRPV) and rabbit oral papillomavirus (ROPV) did not contribute to cross-protective responses between these PV types. Furthermore, Kawana *et al*. (1999) demonstrated that HPV-16 L2 peptide aa 95-107 was not bound by HPV-16 L1/L2 VLP monoclonal antibodies (MAb) and Schellenbacher *et al*. (2009) recently demonstrated that peptides did not elicit neutralisation responses without the aa 113-115 region. Taken together, these studies show HPV-16 L2 aa 96-115 region contains a broadly neutralising epitope, with strong evidence suggesting the epitope occurs at aa 113-115.

Despite these positive results, the HPV-16 L2 aa 108-120 peptide was not cross-protective against diverse PV types CRPV and ROPV rabbits (Embers

et al., 2002), in contrast to a longer L2 aa 11-200 peptide (Gambhira *et al*., 2007a). This suggests there are other cross-neutralising epitopes within the conserved aa 11-107 region. Furthermore, Jagu *et al*. (2009) reported that the HPV-16 L2 peptide containing aa 89-200 did not cross-neutralise several heterologous HPV types, in contrast to the peptides aa 13-107 and aa 12-200 (Jagu *et al*., 2009), suggesting important epitopes may occur between aa 13-89.

(b) L2 epitopes within aa 56-81

Another highly conserved L2 region comprises of aa 56-81. Kawana *et al*. (1998) first identified this epitope, demonstrating that the linear HPV-16 L2 aa 69-81 peptide was bound by 7 of 11 anti-L2 MAb raised against HPV-16 L1/L2 VLPs. The linear peptide containing aa 63-75 was also bound by 1 linear MAb, suggesting that the aa 69-81 region contains the epitope for 6 MAb, and the 7th MAb epitope is contained within aa 69-75. In addition, the peptide containing aa 69-81 cross-reacted with human sera raised against the L1 proteins of HPV-6, 16, 18 and 58 indicating that this region contains a common immunodeterminant which is exposed on the HPV virion surface. The peptide fused to KLH and inserted into BPV-1 L1 produced antisera which neutralised both HPV-16 PsV and HPV-11 authentic virions (Slupetzky *et al*., 2007).

A longer L2 peptide containing aa 64-81 fused to bacterial thioredoxin (Trx) was also shown to be broadly cross-neutralising (Rubio *et al*., 2009). This region was mapped using three HPV-16 L2 peptides: aa 56-71, 61-75 or 64-81 (Kondo *et al*., 2007). All peptide antisera neutralised HPV-16 and cross-neutralised HPV-18 and 58. However, only the aa 56-71 peptide cross-neutralised HPV-31 (Kondo *et al*., 2007; Conway *et al*., 2011), suggesting aa 56-61 are essential for this epitope and aa 75-81 may be redundant. Further work using the L2 aa 56-75 peptide inserted into HPV-16 L1 showed the cross-protection was extended to HPV-52 (Kondo *et al*., 2008). The importance of the region comprising of aa 56-75 was further emphasized by Schellenbacher *et al*. (2009), who demonstrated the L2 aa 75-112 peptide inserted into BPV-1 L1 did not neutralise HPV-16 or other hetrologous types.

(c) L2 epitopes within aa 17-36

The L2 region composed of aa 17-36 has been shown to elicit cross-protection against 10 papillomavirus (PV) types to date, including high-risk types HPV-16, 18, 31, 45, 52, 58, low-risk types HPV-6 and 11, non-related type HPV-5 and the evolutionary divergent type BPV-1 (Gambhira *et al*., 2007b; Alphs *et al*., 2008; Schellenbacher *et al*., 2009). Furthermore, the RG-1 MAb generated against this peptide protected mice in a HPV-16 PsV challenge and depletion of the peptide-specific antibodies reduced or eliminated neutralisation of HPV-16 and 18, further evidence of the importance of this region in neutralisation (Gambhira *et al*., 2007b).

Additionally, the L2 aa 18-38 peptide is cross-protective (Kondo *et al*., 2007; 2008), creating doubts about the necessity of the aa 17 in the epitope. Schellenbacher *et al*. (2009) analysed HPV-16 L2 peptides composed of aa 17-36 or aa 18-31 inserted into the DE loop of BPV-1 L1. In comparison to L2 aa 18-31, the longer peptide (L2 aa 17-36) elicited higher titres of neutralising antibodies against HPV-5, 16, 18, 45 and 58, similar titres to HPV-52 and additionally neutralised HPV-11. This suggests the residues 17 and 32-36 play a role in the cross-neutralisation of several HPV types, particularly HPV-11.

Recent studies have examined the L2 peptide aa 20-38. The peptide was shown to be more effective than five other peptides covering the N-terminal aa 1-120 of HPV-16 L2 in the cross-neutralisation of HPV-18, 31, 45 and 58 (Rubio *et al*., 2009). Two cross-neutralising MAb targeted this peptide and the residues critically involved in binding were identified to be aa 21-30, which overlap the characterized RG-1 epitope and require the two cysteine residues (C22 and C28) to be present for binding (Rubio *et al*., 2011). As a whole, the L2 aa 17-36 epitope appears to have the greatest potential for broad cross-protection.

(d) Other L2 epitopes

Cross-neutralisation has been reported for L2 proteins from other PV types. The BPV-1 L2 peptide containing aa 1-88 has been shown to be

cross-reactive against heterologous PV types CRPV and HPV, specifically HPV-6, 16, 18, 31 and native HPV-11 (Pastrana *et al*., 2005; Gambhira *et al*., 2007a).

1.5.2 L1/L2 chimaeras

A major problem with L2 vaccines is lack of exposure on the surface of mature L1/L2 capsids (Buck *et al*., 2008) and the immunogenic subdominance of L2 (Kirnbauer *et al*., 1992). Although L2 peptide/protein vaccines have been utilized, they have been shown to have low immunogenicity. A strategy to overcome these limitations is the fusion of L2 proteins or epitope peptides with L1 to produce L1/L2 chimaeric proteins.

1.5.2.1 Engineering HPV L1-based vaccines

The success of the L1 VLP vaccines has prompted the development of second-generation VLP-based vaccines incorporating polypeptides. L1 is considered an ideal carrier molecule for several reasons: (a) VLPs elicit immune responses without the use of an adjuvant (Ohlschläger *et al*., 2003; Wakabayashi *et al*., 2002; Reddy *et al*., 2004; Greenstone *et al*., 2008), (b) facilitate display of antigens to both the cellular and humoral arms of the immune system (Lenz *et al*., 2003; Da Silva *et al*., 2001), and (c) tolerate foreign peptides within the surface-exposed areas (Sadeyen *et al*., 2003; Varsani *et al*., 2003a). The L1 structure and the assembly into VLPs has been extensively studied (Chen *et al*., 2000; 2001; Modis *et al*., 2002; Bishop *et al*., 2007a) and several groups have examined the display of peptides in various L1 surface regions to overcome the low immunogenicity of peptide antigens.

1.5.2.2 Structural characteristics of L1

L1 spontaneously forms capsomeres (Rose *et al*., 1993, Modis *et al*., 2002), and self-assembles into virus-like particles (VLPs), which may be either a spherical icosohedral lattice structure of T = 7 symmetry that is morphologically indistinguishable from native HPV virions (Kirnbauer *et al*., 1992, 1993; Hagensee *et al*., 1993; Rose *et al*., 1994a), or a smaller T = 1 “small VLP” particle comprised of 12 L1 pentamers (Chen *et al*., 2000). VLP formation requires inter-capsomer hydrophobic interactions involving the

helices 2, 3, and 4 in the C-terminal region of each L1 monomer (Chen *et al*., 2000; Modis *et al*., 2002; Bishop *et al*., 2007a). The assembly of L1 into the higher-order structures is described in detail by Modis *et al*. 2002 and is shown in Figure 4.

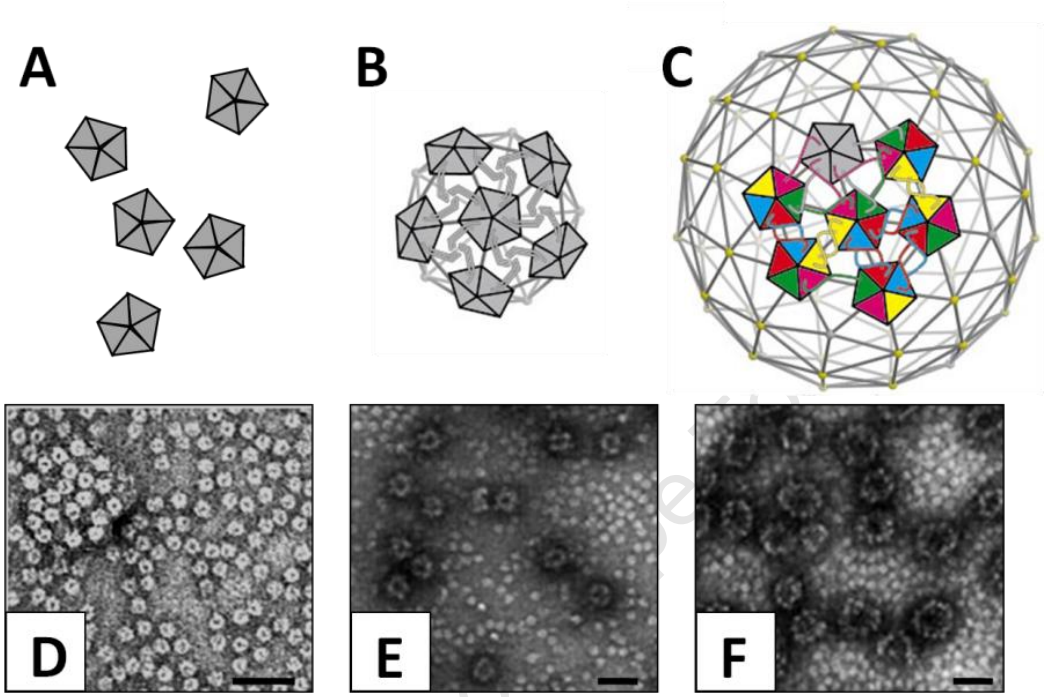

Figure 4: Structural assembly of HPV-16 L1. Schematic diagrams of the L1 pentamer interactions are shown from A-C (modified from Modis *et al*., 2002). A) Individual pentamers consist of five monomeric subunits, represented by grey triangles. B) Assembly of 12 pentamers into a T=1 small VLP occurs by the L1 C-terminal arms folded back toward the L1 monomers. C) Assembly of 72 pentamers into T=7 full-sized VLPs occurs by the L1 C-terminal arms projecting outwards to neighbouring pentamers. Electron micrographs of the structures are shown from D-F (taken from Bishop *et al*., 2007a and Chen *et al*., 2000). D) HPV-16 L1 pentamers (10-20 nm), with an aggregate in the top left corner. E) T=1 small VLPs (35-40 nm), derived from N-terminal truncated HPV-16 L1. F) T=7 VLPs (55-60 nm) derived from full-length HPV-16 L1. Pentamers are also visible in the background of E and F. Scale bar represents 50 nm.

1.5.2.3 Molecular basis of L1 type-specificity

The L1 sequence is highly conserved amongst papillomaviruses (Bishop *et al*., 2007b), suggesting the type-specificity of the L1 immune response is related to the VLP structure (Figure 5). Several highly homologous regions comprise the inner core pentamer structure, and four or more hypervariable regions make up the hypervariable surface loops displayed on the outer face of the capsid (Chen *et al*., 2000).

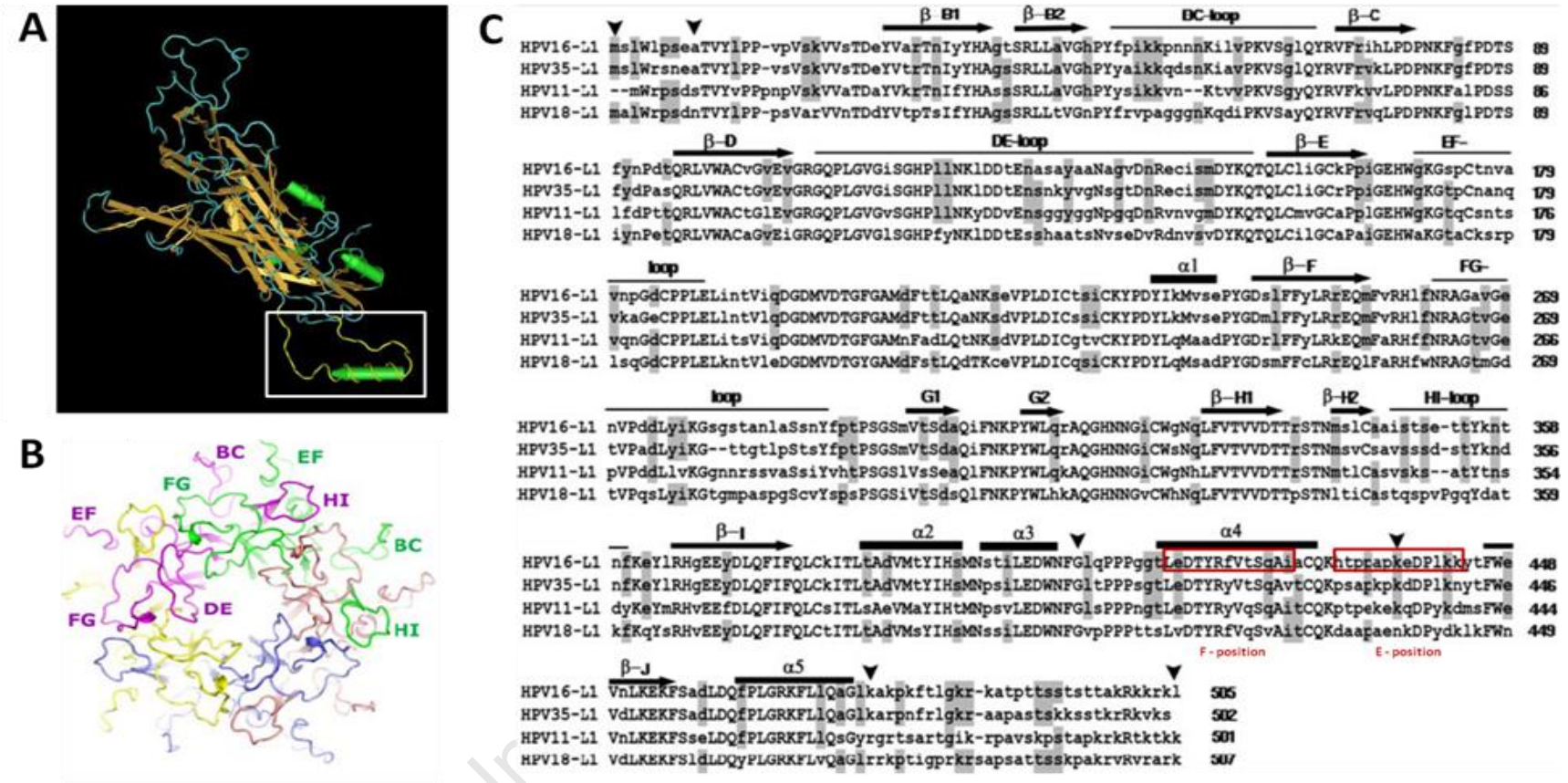

Figure 5: HPV L1 structure and sequences. A) Structural representation of a HPV-16 L1 monomer. The helix 4 domain and the surrounding residues (aa 401 – 439) is indicated with the white box (taken from Murata *et al*., 2009). B) HPV L1 pentamer, comprising of 5 L1 monomers with the surface loops indicated on the diagram (taken from Bishop *et al*., 2007b). C) L1 sequence alignment of L1 from HPV11, HPV16, HPV18, and HPV35. Conserved residues and non-conserved residues are shown in capital letters and lowercase letters respectively. Residues conserved among three HPV types are highlighted in gray shading. The arrows represent β sheets, the α helices indicated with thick bars. The five surface loops are labelled above the sequences (Taken from Bishop et al., 2007b). The F- and E-positions used to create the L1/L2 chimaeras described by Varsani *et al*. (2003a) are indicated with red boxes.

Conformational epitopes, predominantly responsible for the production of NAb (Christensen *et al*., 1994; Roden *et al.*, 1996; White *et al.*, 1998, 1999; Giroglou *et al*., 2001a), are located on the L1 surface loops BC, DE, EF, FG, and HI (Chen *et al.,* 2000; Bishop *et al.* 2007a), specifically the FG and HI loops of HPV-16 (Roden *et al*., 1997; White *et al*., 1999; Christensen *et al*., 2001). In a study done by Bishop *et al*. (2007b), the HPV-11, 16, 18, and 35 L1 pentameric crystal structures revealed distinctive conformational differences in the surface loops containing known neutralising epitopes, thus explaining the type-restricted L1 immune response.

1.5.2.4 Modifications affecting L1 assembly

There are a number of studies describing the use of PV VLPs to deliver and/or display foreign epitopes, either by HPV L1 C-terminal fusion (Windram *et al*., 2008; Müller *et al*., 1997; Kuck *et al*., 2006; Ashrafi *et al*., 2008; Paz De la Rosa *et al*., 2009), or by insertion of epitopes into the core sequences of the HPV-16 L1 (Slupetzky *et al*., 2001; Varsani *et al*., 2003a; Sadeyen *et al*., 2003) with the intention of effectively displaying the peptide epitope on the L1 surface and improving the elicited immune response. The modification of the PV L1 gene and its subsequent affect on L1 immunogenicity and assembly is an important factor to consider in the design of L1-based chimaeras.

Several studies have identified HPV-16 L1 functional domains required for VLP assembly. These are the first 10 residues of the N-terminal (Chen *et al*., 2000, 2001) and the last 30 residues of the C-terminal region, including the nuclear localization signal (NLS) located between aa 484-505 (Zhou *et al*., 2001b; Varsani *et al*., 2006a). The N-terminal truncation has been shown to affect the assembly of VLPs into T=1 or T=7 particles in *E. coli* (Chen *et al*., 2000). The 10 residue N-terminal truncation of HPV-16 L1 forms T = 1 small VLPs, while a 9 residue N-terminal or 30 residue C-terminal truncation does not affect assembly into T=7 particles (Figure 4). C-terminal deletions longer than 30 residues had been reported to render the protein unstable and sensitive to protease degradation (Chen *et al*., 2000).

In addition, the helix 4 (h4) appears to play a role in VLP assembly and is located between residues 414-426 (Varsani *et al*., 2003a). The removal of these motifs results in capsomere formation and prevents further self-assembly into VLPs (Bishop *et al*., 2007a). Varsani *et al*. (2006b) confirmed the importance of h4, as well as the C-terminal residues 428-465, which partially include the h5 helix.

Another important consideration for assembly is the formation of disulphide bonds in higher-order structures. Disulphide bonds are present in T=7 VLPs between the highly conserved cysteine residue 175 and 428 (Li *et al*., 1998; Sapp *et al*., 1998; Fligge *et al*., 2001) via an invading C-terminal arm (Modis *et al*., 2002) and bonds do not occur in T=1 particles, where inter-pentameric interactions is mediated by helices h3, h4 and h5. Mutations of these cysteines results in the formation of capsomeres rather than VLPs (Li *et al*., 1998; McCarthy *et al*., 1998; Sapp *et al*., 1998; Fligge *et al*., 2001; Varsani *et al*., 2006b).

1.5.2.5 L1 surface display sites

Several studies have examined the insertion of epitopes into the PV L1 surface regions with the intention to enhance the immunogenicity of the peptide without compromising the structure and immunogenicity of the L1 VLP. Large regions of the HPV L1 C terminal are surface-exposed and antigenic, especially the loop containing residues 420-429 (Modis *et al*., 2002).

Sadeyen *et al*. (2003) tested the insertion of a short hepatitis B core (HBc) antigen epitope into the surface-exposed loops BC, DE, EF, FG and HI of HPV-16 (Figure 5). The insertions did not affect VLP assembly but diminished the HPV-16 L1 response, particularly within the FG loop (also described by Slupetzky *et al*., 2001). The DE loop in particular has been used by several studies and has shown to elicit both anti-L1 responses and antibodies directed against the inserted peptide (Slupetzky *et al*., 2001). Although VLP assembly is not affected for the majority of constructs (Chackerian *et al*., 1999; Varsani

et al., 2003a; Sadeyen *et al*., 2003; Zhang *et al*., 2004; Schellenbacher *et al*., 2009), the affect may be peptide dependent (Slupetzky *et al*., 2007; Schellenbacher *et al*., 2009).

Varsani *et al*. (2003a) also examined other exposed C-terminal regions for epitope display, using a well-characterized cross-neutralising HPV-16 L2 epitope comprising of aa 108-120 (Kawana *et al*., 1999) whose sequence replaced the h4 region (F-position) and a coil between the h4 region and the β-J sheet (E-position; Figure 5). The chimaeras successfully displayed the L2 peptide, reacted with all L1 MAb and demonstrated both anti-L1 and L2 immune responses, suggesting that both theses insertion sites have potential for epitope display. Despite the disruption of VLP assembly associated with the deletion of h4 (Chen *et al*., 2001; Bishop *et al*., 2007a; Murata *et al*., 2009), the chimaera with L2 in the F-position elicited the highest anti-L2 response, suggesting the peptide was efficiently displayed on the L1 capsomere surface. Although h4 is not a surface loop, structural models predict this region projects outward from capsomeres and is highly exposed on the surface (Murata *et al*., 2009).

The chimaera with the insertion of L2 in the E-position demonstrated lower L2 immunogenicity, as this insertion region is less exposed (Varsani *et al*., 2003a; Kondo *et al*., 2008). However the chimaera was capable of assembling into VLPs and induced higher anti-L1 responses than the chimaera with the h4 insertion. Although the production of neutralising antibodies was not evaluated, the results suggest that chimaeras with epitope insertions in these two regions were the most immunogenic and have potential for the further development of HPV L1-based vaccines.

1.5.2.6 Immunodominance of L1

An important factor to consider in L1-based vaccines is the immunodominance of L1 epitopes, which bias the immune response toward L1 and result in the exclusion of subdominant epitopes. The high immunogenicity appears to be due, at least in part, to the presence of closely

spaced repetitive units of the antigen (Chackerian *et al*., 2002). Importantly, epitope immunodominance may be dependent on its presentation (Sandburg *et al*., 1998) and has been linked to the presence of tertiary or quaternary structures (Ito *et al*., 2003). L1 immunodominant responses have been reported for some chimaeric L1/E7 vaccines (Bian *et al*., 2008; Liu *et al*., 2000), although the immonodominance has not negated humoral or cellular responses to inserted peptides, and studies have shown the immunogenicity of epitope peptides is improved by insertion into L1 (Qian *et al*., 2006; Slupetzky *et al*., 2007)

In summary, vaccination with L2 protects animals from experimental viral challenge and elicited cross-protective responses (Roden *et al*., 2000) mediated by broadly neutralising antibodies that recognize conserved linear epitopes (Gaukroger *et al*., 1996; Pastrana *et al*., 2005). Several cross-neutralising L2 epitopes have been mapped to the first 120 residues of the N-terminal, including HPV-16 L2 aa 90-122, 56-81, 17-36 and BPV-1 aa 1-88. The use of these regions in second generation L1/L2 chimaera vaccines may improve the immunity of L2 and broaden the protection of L1-based vaccines.

1.6 Therapeutic HPV vaccines

The current treatment of HPV-induced lesions by surgical procedures or other methods is both invasive and inefficient (Govan, 2005) and both the current prophylactic vaccines lack therapeutic efficacy (Hildesheim *et al.*, 2007; FUTURE II Study Group, 2007b). In light of the high prevalence of HPV infection and cervical cancer burden worldwide (Muñoz *et al.*, 2004), there is an immediate need for therapeutic HPV vaccines to treat existing HPV-induced neoplastic lesions and induce regression of premalignant cervical dysplasias, a strategy which is less invasive, more specific and potentially more effective.

Protection against the development of HPV-associated disease is thought to be primarily associated with cell-mediated immunity (Scott *et al*., 2001)

and thus an effective therapeutic vaccine requires the induction of strong cell-mediated immune responses against HPV-specific tumour antigens. Therapeutic vaccines have focused on the non-structural oncogenic HPV proteins, particularly E6 and E7, which are responsible for the induction and maintenance of cellular transformation and are constitutively expressed in HPV-infected premalignant and malignant tissues (review by zur Hausen, 2000).

The potential of therapeutic HPV vaccines to induce tumour regression has been tested directly in pre-clinical murine models; using transplantable tumours expressing HPV E7, or by the prevention of tumour formation in challenge experiments (Torréns *et al.*, 2005). Studies have demonstrated that E7 peptides restricted by mouse H2-D^b MHC class I molecules induce tumour regression in mice (Feltkamp *et al.*, 1993; Torréns *et al.*, 2005; Paz De la Rosa *et al.*, 2009), and thus the use of mouse-restricted cytotoxic T-cell lymphocytes (CTL) epitopes in HPV candidate vaccines allows for the preclinical demonstration of therapeutic efficacy against E7-expressing transplantable tumours.

Many HPV candidate vaccines have shown therapeutic efficacy in mice (De Bruijn *et al.*, 1998; Revaz *et al.*, 2001; Ohlschläger *et al.*, 2003), including plant-derived E7-based vaccines (Franconi *et al.*, 2002; Franconi *et al.*, 2006; Massa *et al.*, 2007). However, similar vaccines containing peptides restricted by human MHC class I molecules have not shown clinical success in human tumour regression (Steller *et al.*, 1998; van Driel *et al.*, 1999; Muderspach *et al.*, 2000), suggesting tumour regression in mice is not necessarily indicative of therapeutic efficacy in humans (Frazer *et al.*, 2011).

More than 40 publications describe antigen-specific immunotherapy studies for the therapeutic treatment of persisting HPV infection or malignant lesions in humans (reviewed by Frazer *et al.*, 2011). Four major classes of HPV immunotherapy vaccines have undergone trials: protein vaccines (Gambhira *et al.*, 2006; Van Doorslaer *et al.*, 2010), peptide vaccines (Muderspach *et al.*,

2000), polynucleotide vaccines (Trimble *et al.*, 2009), and recombinant viral vectors (García-Hernández *et al.*, 2006).

The majority of the studies have focused on targeting E6 or E7 proteins and have demonstrated induction of antigen-specific humoral and cellular immune responses (Bodily and Laimins, 2011). Recently, a HPV vaccine has shown significant efficacy in human clinical trials and provided strong evidence of a therapeutic effect, thus stimulating further interest in the field. High-grade vulval intraepithelial neoplasia (VIN3) patients immunised with synthetic long overlapping peptides from HPV-16 E6 and E7, together with incomplete Freund's adjuvant, demonstrated a complete and durable regression of premalignant lesions in 47% of patients, with a further 31% showing a partial response (Kenter *et al.*, 2009). The response was associated with a strong and broad HPV-specific T-cell type 1 (Th1) CD4+ and CD8+ response that peaked after the first vaccination (Kenter *et al.*, 2009, Welters *et al.*, 2010).

1.6.1 HPV-16 E7 cytotoxic T-cell epitopes

Protein-based vaccines are considered safer for clinical application compared with DNA or virus-based vaccines, although there are safety concerns regarding the use of the full length oncogenic proteins (Ohlschläger *et al.*, 2006). The use of specific T-cell epitopes overcomes these concerns and may improve vaccine efficacy by the removal of non-neutralising epitopes, or those which bias the humoral response toward epitopes of limited importance (Cleveland *et al.*, 2000).

Kast *et al.* (1994) identified four HPV-16 E7 human-restricted T-cell epitopes comprising of aa 11-20 (YMLDLQPETT), 12-20 (MLDLQPETT), 82-90 (LLMGTLGIV) and 86-93 (TLGIVCPI). Further work by Ressing *et al.* (1995) indicated three of the peptides (aa 11-20, 82-90 and 86-93) stimulated the lysis of human cancer cells and they have all been subsequently investigated in clinical trials. The aa 11-20 and aa 86-93 epitopes were shown to elicit immune responses but no clinical effects was observed, either when used as individual peptide vaccines (van Driel *et al.*, 1999), by fusion of aa 86-93 to a

universal T-helper epitope (Steller *et al.*, 1998), or by immunization of both aa 12-20 peptides and the aa 86-93 fusion protein (Muderspach *et al.*, 2000).

In addition, Feltkamp *et al.* (1993) identified an E7 CTL epitope specific to H2-D^b allele-restricted mice (binds specifically to H2-D^b MHC class I molecules) which was comprised of aa 49-57 (RAHYNIVTF), and the elicited E7-specific CTL response protected mice from challenge with HPV-16 tumour cells. As a result, this epitope has been used successfully in several tumour regression and CTL response studies in mice (Peng *et al.*, 1998; Liu *et al.*, 2000; Torréns *et al.*, 2005; Daftarian *et al.*, 2006; Kuck *et al.*, 2006; Bian *et al.*, 2008; Paz De la Rosa *et al.*, 2009).

1.6.2 L1/E7 chimaera vaccines

HPV vaccines that are both prophylactic and therapeutic will be particularly beneficial in the treatment and prevention of HPV infections. L1-based chimaeras containing E7 have the potential to be dual vaccines, utilizing the immunogenicity of L1 VLPs to enhance cellular immune responses against E7 epitopes (Jochmus *et al.*, 1999; Rudolf *et al.*, 2001).

Several E7 chimaeric vaccines have been comprised of fusion proteins, whereby either full length or N-terminal regions of E7 are fused to the L1 C-terminal to create L1/E7 chimaeras (Müller *et al.*, 1997; Jochmas *et al.*, 1999; Schafer *et al.*, 1999; Kaufmann *et al.*, 2001; Freyschmidt *et al.*, 2004; Kuck *et al.*, 2006; Kaufmann *et al.*, 2007; Bian *et al.*, 2008), or fused to the C-terminal of L2 to create L1/L2/E7 chimaeras (Greenstone *et al.*, 1998; Rudolf *et al.*, 2001; Wakabayashi *et al.*, 2002; Da Silva *et al.*, 2003; Qian *et al.*, 2006; Xu *et al.*, 2007).

Phase I clinical trials for a L1/E7 fusion chimaera demonstrated the induction of L1 and E7-specific antibodies and cellular immune responses, although clinical efficacy was not significant (Kaufmann et al., 2007). However, both L1/E7 (Kaufmann et al., 2001) and L1/L2/E7 (Rudolf et al., 2001; Warrino *et*

al., 2005) chimaeras induce human cellular immune responses *in vitro*, suggesting further research is needed.

Studies have used E7 CTL epitopes in chimaeric VLPs (Peng *et al.*, 1998; Liu *et al.*, 2000; Paz De la Rosa *et al.*, 2009) and the insertion of the HPV-16 E7 mouse H2-D^b-restricted CTL epitope (aa 86-93) into BPV-1 L1 C-terminal demonstrated efficacy in mice (Peng *et al.*, 1998). Recently, Paz De la Rosa (2009) fused several HPV-16 E6 and epitopes to the HPV-16 L1 C-terminal and the chimaeric VLP (cVLP) induced HPV-specific humoral and cellular immune responses in mice. The E7 epitopes included the mouse-restricted epitope aa 49-57 and the human-restricted epitopes aa 86-93 and aa 37-54 (T-helper epitope).

Taken together, these studies indicate chimaeric VLP-based vaccines containing E7 CTL epitopes are a potential strategy for the development of duel prophylactic and therapeutic HPV vaccines.

1.7 Reducing vaccine costs

The high cost of vaccines has limited their global implementation, particularly in developing countries with the highest cervical cancer burden (Muñoz *et al*., 2004). An attractive approach to reduce the cost of HPV vaccines includes: (a) the use of L1-based capsomere vaccines, and (b) alternative low-cost bacterial or plant expression systems.

1.7.1 Capsomere-based vaccines

The use of L1 capsomere-based vaccines is a potential strategy for the development of thermostable and cost-effective vaccines. Capsomeres induce high titres of anti-L1 neutralising antibodies and T-cell responses (Ohlschläger *et al*., 2003; Dell *et al*., 2006; Thönes *et al*., 2008), effectively display foreign antigens (Varsani *et al*., 2003a; Murata *et al*., 2009) and protect against viral challenge in animal models (Rose *et al*., 1998; Yuan *et al*., 2001). Furthermore, capsomeres are stable at room temperature, thus negating the

need for refrigeration. This could facilitate the introduction of HPV vaccines into low resource settings where they are needed the most.

Although L1 capsomeres induce 20 to 40-fold lower humoral immune responses in comparison to VLPs (Thönes *et al*., 2008), the use of an adjuvant can potentially close this gap (Yuan *et al*., 2001; Schädlich *et al*., 2009; Jagu *et al*., 2010) and both antigens induced similar CD8 T-cell responses after subcutaneous, intranasal, and oral immunization (Thönes *et al*., 2008). As a result, HPV capsomeres show potential as an alternative vaccine antigen.

1.7.2 Plant expression systems

Several hosts have successfully expressed HPV-16 L1, including bacteria (Chen *et al*., 2001), yeast (Carter *et al*., 1991), insect cells (Le Cann *et al*., 1994), mammalian cells (McLean *et al*., 1990) and plants (Biemelt *et al*., 2003; Warzecha *et al*., 2003; Varsani *et al*., 2003b). Cell culture-derived vaccines, such as the current HPV vaccines produced in recombinant baculovirus and yeast systems, are associated with relatively high production costs, require expertise and are difficult to scale up (Tiwari *et al*., 2009; Daniell *et al*., 2009; Demain and Vaishnav, 2009), thus limiting their widespread application in developing countries. Similar problems are associated with mammalian cell systems and thus do not present a cost-effective alternative. Although bacterial systems have potential for low-cost production of antigens, they lack the eukaryotic post-translational machinery which is often necessary for human vaccine antigens, and *E. coli*-expressed HPV L1 exclusively assembles into capsomeres (Li *et al*., 2001; Yuan *et al*., 2001). Moreover, all these systems are amenable to contamination by micro-organisms, endotoxins or pyrogens, thus requiring extensive purification (Demain and Vaishnav, 2009; Tiwari *et al*., 2009).

The use of plant expression systems for the large-scale production of foreign antigens has been proposed as a cost-effective alternative for vaccine production. Plants are easier to cultivate than mammalian cell cultures, the

system allows rapid scale-up and expression of recombinant proteins free of contamination by bacterial toxins or human pathogens (i.e. increased safety) and they contain eukaryotic protein modification machinery, allowing sub-cellular targeting, proper folding, and post-translational modifications (Fischer *et al*., 2004). The ability to rapidly scale-up production according to demand is particularly advantageous for rapid-response vaccine scenarios such as bio-terror threats or emerging diseases (Chichester *et al.*, 2009; Rybicki, 2010).

In particular, it has been proposed that plant production of human and animal vaccines may lower the cost of production significantly, especially for oral vaccination (reviewed by Fischer *et al.*, 2004). However, research is moving away from the original concept of "edible vaccines" as a result of several practical and ethical issues, and it is generally accepted that plant-derived vaccines will need to processed and reproducibly formulated (Rybicki, 2010). Regardless, estimates suggest plants systems reduce vaccine production costs by 31% and thus provide cheaper production alternatives to well-established conventional HPV vaccines and new second-generation HPV vaccines (Rybicki, 2009; Rybicki, 2010).

A variety of plants have been used to express human and animal vaccine antigens (detailed reviews by Rybicki, 2009; Rybicki, 2010; Lössl and Waheed 2011). Plant-produced candidate vaccines have generated antigen-specific systemic and mucosal immune responses and conferred protection against challenge in animal model systems (Streatfield and Howard, 2003). Several vaccines are at different stages of clinical trials (Rybicki, 2010; Obembe *et al*., 2011) and two plant-derived proteins have been licenced (Website 1: http://www.molecularfarming.com): a transgenic tobacco-produced antibody (Pujol *et al.*, 2005) and an injectible veterinary NDV vaccine for poultry (Website 2: http://www.dowagro.com/; Rybicki, 2009).

A practical limitation of plant systems is low yields of recombinant protein, potentially a result of protein instability or low-level expression. There are several key economic considerations and approaches to improving transcript

expression and protein stability (reviewed by Fischer *et al.*, 2004; Schillberg *et al.*, 2005; Obembe *et al.*, 2011). Strategies include the use of strong plant promoters, codon-optimization of the gene and introduction of a 5'-untranslated plant virus sequence or other translational enhancers. Furthermore, the co-expression of viral silencing suppressor proteins (Takeda *et al.*, 2002; Voinnet *et al.*, 2003) and the use of transcription factors (Yang *et al.*, 2001) can further enhance transcript stability and transgene expression.

The stability of recombinant proteins has been considered as the single most important factor limiting the yields in plant production systems (Schillberg *et al.*, 2005) and poor protein folding and the resultant instability reduces the accumulation of heterologous proteins in plants (Ma *et al.*, 2003). This problem has been effectively addressed by targeting the proteins to the secretory pathway (Franconi *et al.*, 2006; Noris *et al.*, 2011) or intra-cellular organelles (Maclean *et al.*, 2007).

Plant expression of recombinant proteins occurs as a result of the genetic transformability of plants (Bevan *et al.*, 1983). Plant expression systems effective in the industrial-scale production of vaccines include: (a) stable transgenic or transplastomic expression in plants, (b) transient expression in plant virus-based or *Agrobacterium tumefaciens*-based plant systems, and (c) seed expression systems (reviewed in Rybicki, 2009).

The two main strategies employed in the production of plant-derived HPV vaccine antigens are transgenic and transient expression. Several groups have expressed HPV-16 L1 VLPs in plants (Appendix A) using transient (Varsani *et al*., 2006a; Maclean *et al*., 2007; Regnard *et al*., 2010) and transgenic plant expression systems (Biemelt *et al*., 2003; Varsani *et al*., 2003b; Liu *et al*., 2005; Fernández-San Millán *et al*., 2008; Lenzi *et al*., 2008; Waheed *et al*., 2011). Other HPV proteins such as CRPV and ROPV L2 (Palmer *et al*., 2006), HPV-8 E7 (Noris *et al*., 2011) and HPV-16 E7 (Franconi *et al*., 2002, 2006; Massa *et al*., 2007; Venuti *et al*., 2009) have also been expressed in plants.

Plant-derived PV L1 and chimaeras of L1 self-assemble into higher-order structures, which are immunogenic and protective in animal models, thus demonstrating proof of efficacy. Furthermore, the first L1/E6/E7 chimaera expressed in plants has been recently described by Paz De la Rosa *et al*. (2009). Immunisation with the plant-derived cVLP demonstrated both antibody and CTL responses in mice, providing evidence of a successful dual HPV prophylactic / therapeutic candidate vaccine.

There is a definitive trend toward the use of transient expression systems for the production of plant-expressed vaccine antigens (Rybicki, 2010), with several advantages including the rapid production of recombinant proteins days after molecular cloning (in comparison to several months required for transgenic regeneration), plants are unaffected by chromosome position effects due to transgene insertions, the system can be used in fully differentiated plant tissues and, most importantly, significantly higher protein levels have been obtained in comparison to stable nuclear transformation (Kapila *et al.*, 1997; Varsani *et al.*, 2006a; Fischer *et al.*, 2004).

Small-scale agroinfiltration using syringe injection has proved particularly useful in the optimisation of transient expression levels, as several gene constructs utilizing different gene modifications and sub-cellular targeting sequences can be simultaneously expressed and analysed (demonstrated by Maclean *et al.*, 2007; reviewed by Rybicki, 2010). In addition, vacuum infiltration is useful in the large-scale production of proteins, allowing expression to be scaled up to industrial levels. This has been demonstrated by Medicago Inc. in Canada (Yusibov and Rabindran, 2008) and the Fraunhofer Institute for Molecular Biology and Applied Ecology and the Institute for Molecular Biotechnology at the RWTH Aachen University in Germany (Fischer *et al.*, 2004). As a result, *Agrobacterium*-mediated transient expression systems have proved useful in both developmental research and in industrial applications.

In conclusion, plant expression systems present an attractive cost-effective alternative for the production of immunogenic PV vaccines. Although

transgenic systems have been traditionally used for HPV-16 L1 expression, transient expression systems are more suitable for the rapid optimisation of protein expression in plants, a necessary process as the expression of recombinant proteins in plants appears to be empirical (Rybicki, 2009).

1.8 Study objectives

The first objective was to broaden the cross-protection of HPV L1-based prophylactic vaccines by creating four L1/L2 chimaera proteins containing a conserved cross-neutralising L2 epitope within HPV-16 L1. The L2 sequences replace the helix 4 (h4) sequence of HPV-16 L1, as this position has shown to induce high anti-L1 and L2 responses (Varsani *et al.*, 2003a). The four L2 epitopes are HPV-16 L2 aa 108-120 (Kawana *et al.*, 1999), aa 56-81 (Kawana *et al.*, 1998), aa 17-36 (Gambhira *et al.*, 2007b), as well as BPV-1 L2 aa 1-88 (Pastrana *et al.*, 2005).

The second objective was to improve the therapeutic efficacy of HPV L1-based vaccines by incorporating HPV-16 E7 CTL epitope sequences into the helix 4 of HPV-16 L1 to create two L1/E7 chimaeras. The two HPV-16 E7 CTL epitopes used in this study are the mouse H2-D^b-restricted E7 CTL epitope comprised of aa 49-57 (Feltkamp *et al.*, 1993), which can be used to investigate tumour regression and CTL responses in mice, and the human HLA-A2-restricted E7 CTL epitope comprised of aa 86-93 (Kast *et al.*, 1994). Another two L1/L2/E7 chimaera candicate vaccines were designed, consisting of HPV-16 L1 containing the HPV-16 L2 epitope aa 108-120 located in the helix 4, and either the mouse or human-restricted E7 CTL epitope in the coil between the helix 4 and β-J sheet, another insertion site described by Varsani *et al*. (2003a) which is useful for the display of foreign epitopes.

The third objective was to optimize the *Agrobacterium-mediated* transient expression of the eight HPV candidate vaccines in *Nicotiana benthamiana*, either by targeting the protein to the chloroplast (Maclean *et al.*, 2007) or by utilizing a self-replicative BeYDV-based vector (Regnard *et al.*, 2010), both strategies which have produced high yields of HPV-16 L1 in plants but have

not been comparatively analyzed. All genes were human codon-optimised for high-level expression as described by Maclean *et al.*, (2007) and the yields were quantified to determine the economic feasibility of using a tobacco transient expression system for HPV chimaera vaccine production.

The fourth objective was to purify three plant-derived L1/L2 chimaeras from plants and examine the immunogenicity of the prophylactic L1/L2 chimaera candidate vaccines in mice. The elicited anti-L1 and L2 response was analysed and the cross-protective potential of the vaccines was determined using HPV-16, 18, 45 and 52 pseudovirion neutralisation assays.

Finally, the study examined the transgenic expression of three HPV chimaeras: L1/L2, L1/E7M and L1/L2/E7M. The different chimaeras either contained the HPV-16 L2 epitope aa 108-120, the mouse-restricted HPV-16 E7 CTL epitope comprising of aa 49-57, or both epitopes. These chimaeras were chosen to directly compare the effect of the L2 and E7 epitope insertions on chimaera expression. All chimaeras were targeted to the chloroplast and the expression of the three chimaeras was compared to yields obtained using a transient expression system.

Chapter 2: Transient plant expression of L1 chimaeras

2.1 Introduction

Cervical cancer is primarily caused by HPV infection and is the third most common cancer among women worldwide (Ferlay *et al*., 2010). As a result, HPV vaccine development is a priority for preventative cancer research. The L1 major capsid protein is the antigen of choice for prophylactic vaccines, as it is immunodominant and self-assembles into VLPs which are structurally and immunologically similar to authentic virions. Vaccination with VLPs elicits high titres of neutralisation antibodies (NAb) in both animals and humans and two multivalent HPV L1 VLP-based prophylactic vaccines have been licensed and are highly effective in the prevention of vaccine-type HPV-16 and 18 infections and associated disease (Schiller *et al*., 2008).

Despite the high efficacy of current HPV vaccines, the type-specificity (Brown *et al*., 2009; Wheeler *et al*., 2009), the lack of therapeutic efficacy (FUTURE II Study Group, 2007b; Hildersheim *et al*., 2007) and the high cost of vaccines (Schiller *et al*., 2008) have limited their widespread application, particularly in developing countries with >80% of the cervical cancer burden (Parkin and Bray, 2006). Therefore, there is an urgent need for affordable second-generation HPV vaccines, which broaden protection to include multiple oncogenic HPV types, and improve the therapeutic efficacy to clear established HPV infections and cancerous lesions.

Broad-spectrum prophylactic HPV vaccines can be developed using cross-neutralising L2 epitopes. The L2 epitopes can be incorporated into surface regions of L1 to create L1/L2 chimaeras displaying the L2 peptide on the surface of assembled L1. Three HPV-16 L2 cross-neutralising epitopes are of interest: aa 108-120 (Kawana *et al*., 1999, 2003), aa 56-81 (Kawana *et al*., 1998; Slupetzky *et al*., 2007; Kondo *et al*., 2007, 2008) and aa 17-36 (Gambhira *et al*., 2007b; Kondo *et al*., 2007, 2008). Furthermore, Bovine papillomavirus type 1 (BPV-1) aa 1-88 has also been shown to elicit

cross-neutralising responses (Pastrana *et al*., 2005; Gambhira *et al*., 2007a) and was included in this study.

The E7 non-structural protein is constitutively expressed in HPV-infected cells and elicits cellular immune responses against HPV-infected tumour cells (zur Hausen, 2000). Several HPV-16 E7 cytotoxic T-cell lymphocyte (CTL) epitopes have been identified (Feltkamp *et al.*, 1993; Kast *et al.,* 1994), and have demonstrated the regression of tumours in animals. As a result, the inclusion of these CTL epitopes within L1 may improve the cellular immunity of HPV vaccines and provides vaccines which are both prophylactic and therapeutic (Müller *et al.*, 1997; Jochmus *et al.*, 1999; Schafer *et al.*, 1999; Kaufmann *et al.*, 2001).

The use of plant expression systems for the large-scale production of foreign antigens has been proposed as a cost-effective alternative for vaccine production (Fischer *et al.*, 2004), with a definitive trend toward the use of transient expression for high-level protein expression and optimisation (Rybicki, 2009). The expression of papillomavirus antigens in plants is of particular interest in this study, specifically HPV-16 L1. Several groups have expressed HPV-16 L1 in plants (Appendix A), as well as HPV-11 L1 (Warzecha *et al*., 2003; Kohl *et al*., 2007), canine oral papillomavirus (COPV) L1 (Azhakanandam *et al*., 2007), and cottontail rabbit papillomavirus (CRPV) L1 (Kohl *et al*., 2006).

The transient expression of recombinant proteins in plants occurs either via plant tissue infiltration of *Agrobacterium tumefaciens* containing transgenes inserted into the transfer DNA (T-DNA) region of the Ti plasmid, or by use of infective virus-based vectors (Rybicki, 2009). A coupled method has recently been developed, whereby viral vectors are integrated into modified *A. tumefaciens* Ti plasmids for the *Agrobacterium*-mediated delivery and release of a replicating viral vector into the host cell (Gleba *et al*., 2005; Rybicki, 2010). Successful amplification vectors have been derived from plant single-stranded DNA geminiviruses such as Bean yellow dwarf virus (BeYDV)

(Halley-Stott *et al*., 2007) and BeYDV-derived DNA replicon systems have shown rapid high-level transient expression for several vaccine antigens in *N. benthamiana*, including Norwalk virus capsid protein (NVCP), hepatitis B core antigen (HBc), HIV-1 p24 and HPV-16 L1 (Huang *et al*., 2009; Regnard *et al*., 2010).

A practical limitation of plant systems is low yields of recombinant protein, potentially a result of protein instability or low-level expression (Fischer *et al.*, 2004; Obembe *et al.*, 2011). It is estimated that plant-expressed recombinant protein yields need to be greater than 1% of the total soluble protein (TSP) to be economically viable (Fischer *et al*., 2004). This is particularly problematic for the expression of recombinant proteins using nuclear-transformed transgenic plants, as these systems are often associated with low yields of recombinant protein (Rybicki, 2009).

HPV-16 L1 has been expressed transgenically in nuclear-transformed potato and tobacco plants (Biemelt *et al*., 2003; Varsani *et al*., 2003b; Liu *et al*., 2005) and transiently in tobacco (Varsani *et al*., 2006a). Although transient expression of HPV-16 L1 using a simple tobacco mosaic virus (TMV)-derived vector (Varsani *et al*., 2006a) improved HPV-16 L1 yields 10-fold in comparison to previous transgenic studies (Varsani *et al*., 2003b), low expression levels of HPV-16 L1 (<1% TSP) were consistently reported for all studies (Appendix A) and the elicited immune responses were relatively weak.

Human codon-optimisation of the L1 gene and targeting to the chloroplast have significantly improved HPV-16 L1 expression in both transgenic and *Agrobacterium*-mediated transient tobacco expression systems (Maclean *et al*., 2007). The higher accumulation of chloroplast-localised L1 in comparison to other intra-cellular compartments has also been demonstrated using transiently-expressed COPV L1 (Azhakanandam *et al*., 2007). Transient expression of the human codon-optimised HPV-16 L1 gene demonstrated >8500-fold increase in yield in comparison to previous TMV-based studies (Varsani *et al*., 2006a), obtaining 340 - 380 mg/kg plant tissue (Maclean *et al*.,

2007; Regnard *et al*., 2010). Expression levels were further improved by use of a self-replicative BeYDV-derived expression vector (Regnard *et al*., 2010), or by targeting the protein to the chloroplast (Maclean *et al*., 2007), to give the highest yields thus far in a tobacco transient expression system (530 - 550 mg/kg plant tissue; 17% TSP).

A recent development in plant-derived HPV vaccines was the expression of the first HPV-16 L1 chimaera in plants. The L1/E6/E7 chimaera consisted of HPV-16 L1 C-terminally fused to several E6 and E7 epitopes and it was expressed in transgenic tomatoes (Paz De la Rosa *et al*., 2009). Although yields were low (0.05 – 0.1% TSP), the chimaera successfully assembled into immunogenic VLPs and demonstrated both antibody and CTL responses in mice, providing evidence of a successful dual HPV prophylactic / therapeutic vaccine.

In addition, other viral coat fusion proteins containing HPV-16 L2 and E7 epitopes have recently been expressed in plants. Cerovská *et al*. (2008) demonstrated a potato virus A coat protein (ACP) fused to a HPV-16 L2 epitope (aa 108-120) and E7 epitope (aa 44-66) correctly assembled into ACP VLPs and the L2 epitope was detected on the surface of the VLPs. Furthermore, Morgenfield *et al*. (2009) expressed a fusion protein consisting of potato virus X (PVX) coat protein and HPV-16 E7 in transformed chloroplasts. These studies show promise for the development of HPV chimaera vaccines which display epitopes on the surface of a carrier protein such as HPV-16 L1.

In conclusion, L1-based chimaeric proteins containing cross-neutralising L2 epitopes and therapeutic E7 CTL epitopes may broaden the protection and improve the therapeutic efficacy of current HPV L1 VLP vaccines. Plant expression systems present a cost-effective alternative for the production of HPV vaccines and plant-derived HPV L1 and L1-based chimaeras self-assemble into immunogenic VLPs. HPV-16 L1 yields were significantly improved by human codon-optimisation of the HPV-16 L1 gene and targeting

the protein to tobacco chloroplasts in *Agrobacterium*-mediated transient expression systems. Similar high yields were obtained using the agroinfiltration-delivered self-replicative BeYDV-derived expression vector.

In this study, eight HPV L1-based chimaeras were expressed in tobacco using an *Agrobacterium*-mediated transient system. Injection agroinfiltration was used for the rapid optimisation of protein expression and several methods were analysed to obtain commercially-viable yields in plants.

2.2 Materials and Methods

2.2.1 Plant expression vectors

Three binary *Agrobacterium* plant expression vectors were used to optimize HPV chimaera expression: pTRAc and pTRAkc-rbcs1-cTP (provided by Prof. Rainer Fischer; Fraunhofer Institute for Molecular Biology and Applied Ecology, Germany) and the Bean yellow dwarf geminivirus (BeYDV) vector pRIC3 (created by Richard Halley-Stott). Two are non-replicative vectors which target the expressed protein to either the cytoplasm (pTRAc) or chloroplast (pTRAkc-rbcs1-cTP) (Maclean *et al*., 2007), and the third is a self-replicating cytoplasm-targeting vector (pRIC3). The pRIC3 vector is a third-generation pRIC vector (Regnard *et al*., 2010), which has been reduced in size and has shown similar amplification of transgene expression *in planta*.

The vectors contain a number of elements necessary for protein expression in plants (Figure 1). The pTRAkc-rbcs1-cTP vector (Figure 1A) is a derivative of pTRAc (Figure 1B), and contains the chloroplast-transit peptide sequence of the potato *rbcS1* gene. The pRIC3 (Figure 1D) contains the BeYDV replication-associated proteins necessary for self-replication (Regnard *et al*., 2010).

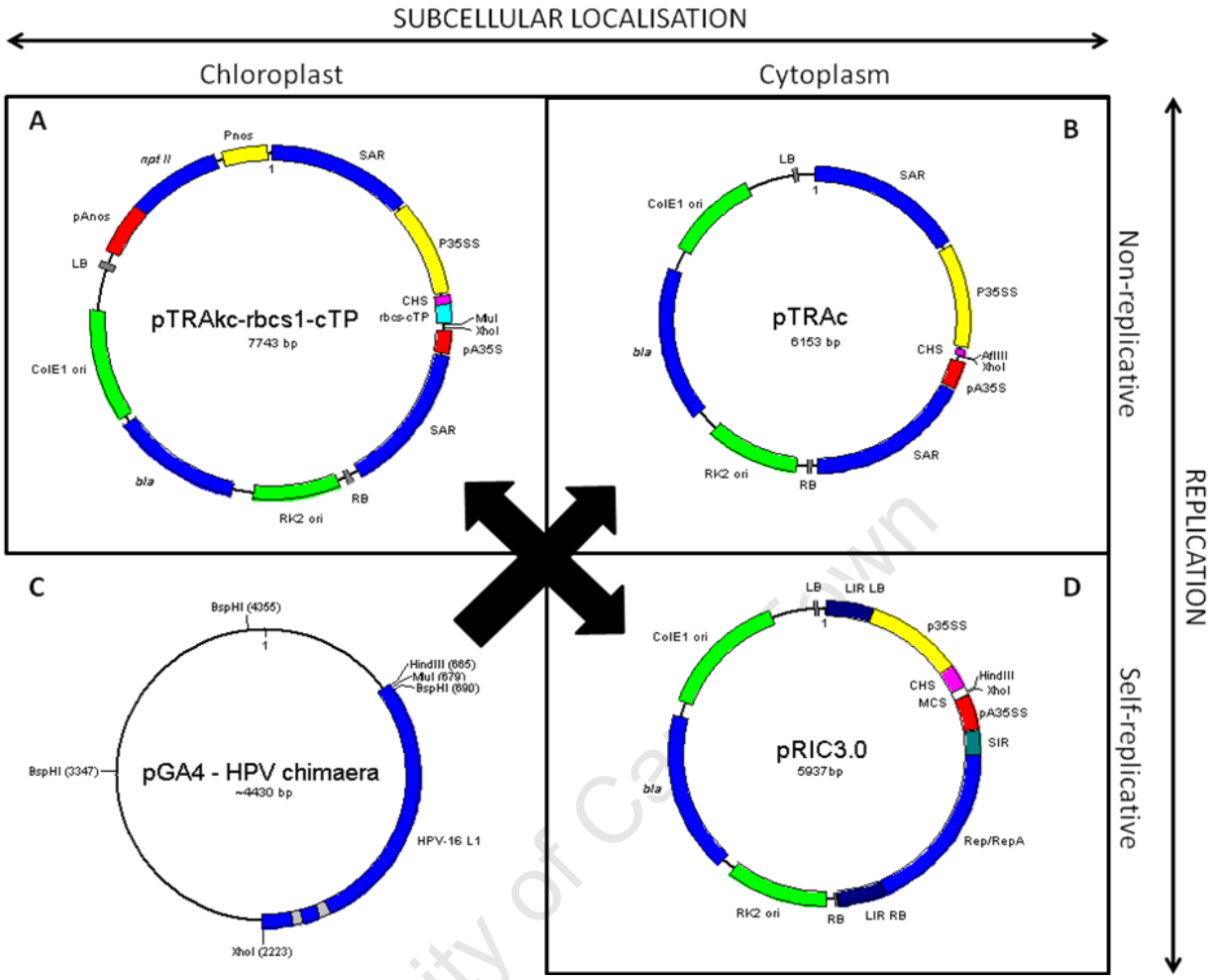

Figure 1: Plasmids used to create the HPV chimaera plant expression constructs. C) HPV chimaera genes from pGA4 constructs were directionally subcloned into the *Agrobacterium* plant expression vectors: A) pTRAkc-rbcs1-cTP, B) pTRAc and D) pRIC3. The vector elements necessary for plant expression are shown in the figure. P35SS: CaMV 35S promoter containing duplicated transcriptional enhancer, CHS: chalcone synthase 5' untranslated region, pA35S: CaMV 35S polyadenylation signal for foreign gene expression, ColE1ori: *E. coli* origin of replication, RK2ori: *Agrobacterium* origin of replication, *bla*: ampicillin / carbenicillin-resistance gene, and LB/RB: left and right borders for T-DNA integration. The pTRAc vector contains SAR: tobacco Rb7 scaffold attachment regions flanking the expression cassette. In addition, the pTRAkc-rbcs1-cTP vector contains n*pt II*: the kanamycin-resistant gene, Pnos/pAnos: promoter / polyadenylation signal of the nopaline synthase gene and rbcs1-cTP: *Solanum tuberosum* chloroplast-transit peptide sequence of the Rubisco small-subunit gene *rbcS1*. The pRIC3 vector contains LIR: BeYDV long intergenic region, SIR: BeYDV short intergenic region, and Rep/RepA: BeYDV rep gene.

2.2.2 Synthesis of the L1 chimaeras

Three types of HPV-16 chimaeras were analyzed: the L1/L2, L1/E7 and L1/L2/E7 chimaeras. The structure of these chimaeras is shown in Figure 2. The chimaeras consist of a South African HPV-16 L1 isolate gene sequence (SALI: GenBank accession no. AY177679) with an L2 or E7 epitope located in the h4 helix at aa 414, or in the coil between the h4 helix and ß-J structural region at aa 433/434 (denoted the “F-position” and “E-position” respectively by Varsani *et al.*, 2003a).

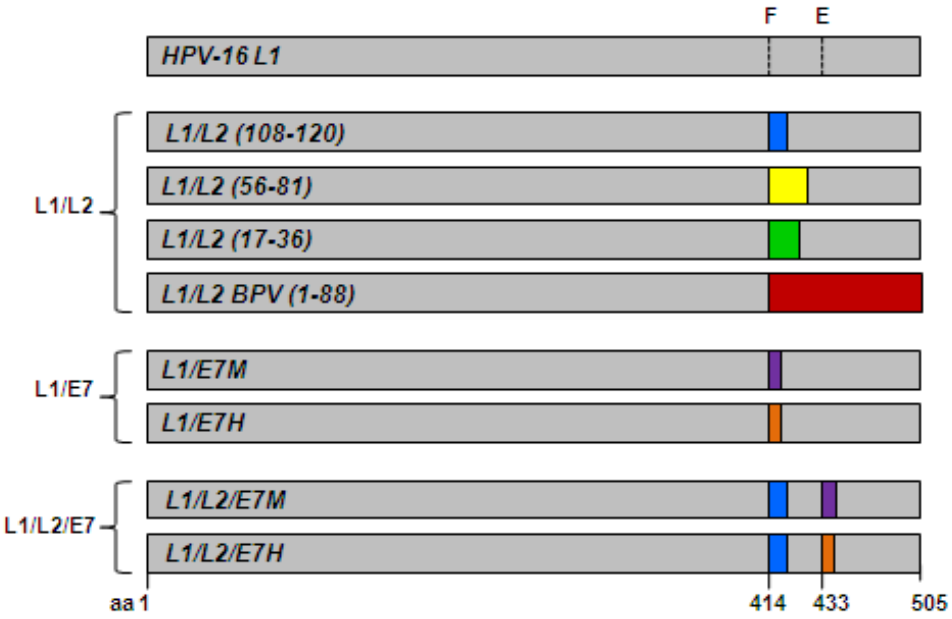

Figure 2: The amino acid location of the HPV L2 and E7 epitopes in HPV-16 L1 for L1/L2, L1/E7 and L1/L2/E7 chimaeras. The L1 C-terminal region containing the h4 helix (F-position) and coil between the h4 and the ß-J structural region (E-position) was targeted in the production of the chimaera genes.

The eight HPV-16 L1 chimaeras used in this study are described in Table 1. These chimaeric genes were designed by Dr Inga Hitzeroth (Plant Vaccine Group, UCT), human codon-optimised and synthesized *in silico* by GENEART AG (Regensburg, Germany) using high throughput gene assembly. Synthesized L2 or E7 epitope sequences replaced the L1 sequence in the E- and F-positions and were not simply inserted into the L1 protein.

Table 1: Summary of the HPV-16 L1 chimaeric constructs

Construct	Inserted epitope	L1 position of epitope		Sequence substitution (aa)
L1/L2(108-120)	HPV-16 L2 aa 108-120	F-position	aa 414-426	13
L1/L2(56-81)	HPV-16 L2 aa 56-81		aa 414-439	26
L1/L2(17-36)	HPV-16 L2 aa 17-36		aa 414-433	20
L1/L2 BPV(1-88)	BPV-1 L2 aa 1-88		aa 414-505	88
L1/E7M*	HPV-16 E7 aa 49-57	F-position	aa 417-425	9
L1/E7H**	HPV-16 E7 aa 86-93		aa 417-424	8
L1/L2/E7M*	HPV-16 L2 aa 108-120	F-position	aa 414-426	13
	HPV-16 E7 aa 49-57	E-position	aa 433-441	9
L1/L2/E7H**	HPV-16 L2 aa 108-120	F-position	aa 414-426	13
	HPV-16 E7H aa 86-93	E-position	aa 434-441	8

* M = mouse-restricted E7 CTL epitope,
**H = human-restricted E7 CTL epitope,

2.2.3 Subcloning of the L1 chimaera genes

The HPV-16 L1/L2, L1/E7 and L1/L2/E7 chimaera sequences were excised from pGA4 vectors using 3' *XhoI* and either 5' *BspHI*, *MluI* or *HindIII* restriction enzyme (RE) sites that flank the chimaeric genes (Figure 1C). The HPV genes were directionally subcloned into the plant expression vectors, using *AflIII* and *XhoI* for pTRAc (Figure 1B), *MluI* and *XhoI* for pTRAkc-rbcs1-cTP (Figure 1A), and *HindIII* and *XhoI* for pRIC3 (Figure 1D). DH5-α chemically competent *E. coli* cells (E.cloni^{TM}, Lucigen) were transformed with the chimaera plasmid constructs and recombinants were selected using ampicillin resistance (100 µg/ml). The pTRAc HPV-16 L1/L2 chimaera constructs L1/L2(108-120), L1/L2(56-81) and L1/L2(17-36) were provided by Mark Whitehead (Plant Vaccine Group, UCT). The plasmid constructs used in this study are summarized in Table 2.

Table 2: *Agrobacterium* expression constructs used in this study

Plant Expression Vector	Chimaeras tested	Plasmid replication	Subcellular localisation	Source
pTRAc	L1/L2	Non-replicative	Cytoplasm	M. Whitehead
pTRAkc-rbcs1-cTP	L1/L2 L1/E7 L1/L2/E7	Non-replicative	Chloroplast	This study
pRIC3	L1/L2 L1/E7 L1/L2/E7	Self-replicative	Cytoplasm	This study

2.2.4 Identification of recombinant L1 chimaeras

L1 chimaera recombinant clones were screened by colony PCR, using pTRAc vector-specific primers and chimaera-specific primers binding to different L2 or E7 epitopes (Table 3). PCR was performed using GoTaq Flexi DNA Polymerase kit (Promega) as per the manufacturer's instructions using 1 µM of each primer in a final $MgCl_2$ concentration of 3 mM.

Table 3: Primers used in PCR and sequencing of the HPV chimaeras

Primer target	Chimaera detected	Primer name	Primer sequence	PCR product (kb)
pTRAc vector	All chimaeas	pTRAc Fwd pTRAc Rvs	5'-CATTTCATTTGGAGAGGACACG-3' 5'-GAACTACTCACACATTATTCTGG-3'	~1.7
L1/L2 chimaeras	All L1/L2 chimaeras	ModNew Fwd	5'-CGACGACCTGTACATCAAGG-3'	-
	L1/L2(108-120) L1/L2(56-81) L1/L2(17-36) L1/L2 BPV(1-88)	VEET Rvs SAF2 Rvs QLYK Rvs SALIBPV Rvs	5'-GATGAAGCTGGTCTCCTCC-3' 5'-GGATGTAGCCGGTCCTGC-3' 5'-ACCTTGGGGATGATGTCAGG-3' 5'-TATCTAGGGCTTCCTCCAGC-3'	0.41 0.44 0.44 0.56
E7 chimaeras	L1/E7M L1/L2/E7M	RAHY Fwd	5'-CCACTACAACATCGTGACCTTC-3'	0.22 0.17
	L1/E7H L1/L2/E7H	TLGI Fwd	5'-CTGGGCATCGTGCCCTATC-3'	0.23 0.18
	All E7 chimaeras	EndMod Rvs	5'-CATCACAGCTTCCGTTTCTTCC-3'	-

2.2.4.1 Colony PCR using vector-specific primers

The pTRAc vector-specific primers (designed by Mark Whitehead) bind upstream and downstream of the multiple cloning site (MCS) to detect the gene insertions. The PCR profile consisted of an initial denaturation step at 95°C for 3 min, followed by 25 cycles at 95°C for 30s, 59°C for 30s and 72°C for 3 min, and a final elongation step at 72°C for 3 min. PCR products were separated on a 0.8% TBE agarose gel and detected using ethidium bromide.

2.2.4.2 Colony PCR using epitope-specific primers

HPV L2 or E7 epitope-specific primers (designed by Marieta Burger) were used to verify the correct chimaera insert in recombinant pTRAkc-rbcs1-cTP and pRIC3 clones. The PCR profile consisted of an initial denaturation step at 95°C for 2 min, followed by 25 cycles at 95°C for 30s, 55°C (L1/L2 chimaeras) or 61°C (E7 chimaeras) for 20s and 72°C for 30s, and a final elongation step

at 72°C for 3 min. PCR products were separated on a 1.2% TBE agarose gel and detected using ethidium bromide.

2.2.4.3 Restriction enzyme digestion

Recombinants were verified by restriction enzyme digestion using RE sites which flank the 1.5 kb chimaera gene insert (*Eco*RI / *Xho*I for pTRAkc-rbcs1-cTP clones, or *Hind*III / *Xho*I for pRIC3 clones). Recombinant DNA (~500 µg) was digested for 1-2 hrs at 37°C, using 1U enzyme per reaction as per manufacturer's instructions (Roche/Fermentas). Digested DNA was separated on a 0.8% TBE agarose gel and stained with ethidium bromide.

2.2.4.4 Sequencing of L1 chimaeras

The HPV chimaera gene insert in pTRAkc-rbcs1-cTP recombinants were sequenced using the pTRAc vector-specific primers. Sequences were aligned with the HPV chimaera sequences using DNAMAN multiple alignment software.

2.2.5 *Agrobacterium* transformation

Agrobacterium tumefaciens GV3101::pMP90RK cells were made electrocompetent using the method described by Shen and Forde (1989). Transformation of *Agrobacterium* was performed as described by Maclean *et al.* (2007) and recombinant clones were screened by antibiotic selection (50 µg/ml Carbenicillin, 50 µg/ml Rifampicin and 30 µg/ml Kanamycin). Successful transformation was confirmed by colony PCR and restriction enzyme digestion (as described in Section 2.2.4).

2.2.6 Agroinfiltration of *N. benthamiana*

A. tumefaciens recombinant chimaera cultures, as well as *A. tumefaciens* LBA4404 cultures containing the pBIN-NSs plasmid encoding the tomato spotted wilt virus (TSWV) NSs silencing suppressor (Takeda *et al*., 2002), were prepared for infiltration as described by Maclean *et al.* (2007). The

Agrobacterium cells were diluted in infiltration media (10 mM $MgCl_2$, 10 mM MES, 3% sucrose and 150 µM acetosyringone in water, pH 5.6) to give a final OD_{600} of 0.25 for individual *Agrobacterium* chimaera strains and a combined OD_{600} of 0.5 for the constructs co-infiltrated with *A. tumefaciens* LBA4404 (pBIN-NSs). The strains were incubated at 22°C for 2 hrs to allow for expression of the *vir* genes prior to infiltration.

Six-week old *N. benthamiana* leaves were agroinfiltrated by injecting the bacterial suspension into the abaxial air spaces from the ventral side of the leaf (Maclean *et al.*, 2007). The plants were grown under conditions of 16 hr light, 8 hr dark at 22°C for the desired time period. Chimaera expression time trials were conducted 1-9 days post-infiltration (dpi), and chimaeras were either co-expressed with or without the NSs silencing suppressor. Separate plants were used for each chimaera, and separate leaves on the same plant were infiltrated with either pTRAc, pTRAkc-rbcs1-cTP or pRIC3 chimaera constructs for the comparative vector expression.

2.2.7 Protein extraction from plants

Leaf discs, cut using the cap of an eppendorf tube, were harvested from agroinfiltrated leaves (~10 mg per disc, 3 discs per construct) and ground in liquid nitrogen. Leaf material was resuspended in 250µl per disc of 1.5M NaCl high salt PBS (HS PBS) extraction buffer containing protease inhibitor (EDTA-free Complete Protease Inhibitor; Roche). The crude plant extract was clarified twice by centrifugation at 13,000 rpm for 5 min and the supernatant was stored at -20°C.

2.2.8 Western blot detection of plant-expressed L1 chimaeras

The plant extracts were incubated at 95°C for 5 min in loading buffer (Sambrook *et al.*, 1989), separated by a 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane by semi-dry electroblotting. The membrane was blocked in blocking buffer for 30 min at room temperature (5% skim milk, 0.1% Tween-20 in 1x PBS, pH 7.6) and incubated overnight at 4°C in anti-L1

primary antibody, diluted in blocking buffer. HPV-16 L1 protein was detected with either mouse monoclonal (MAb) CamVir1 (1:10000; Abcam, UK), which binds to the L1 linear epitope GFGAMDF located at aa 230-236 (McLean *et al*, 1990), or H16.J4 (1:2500) which binds a linear epitope located at aa 261-280 within the FG loop of the L1 protein (Christensen *et al*., 1996). Both binding sites are not destroyed by the L2 or E7 epitope insertions.

Membranes were washed with blocking buffer for 4x 15 min, and incubated in secondary goat-anti-mouse-alkaline phosphatase conjugate (1:10000; Sigma) diluted in blocking buffer for 2 hrs at room temperature. Membranes were finally washed with wash buffer (0.1% Tween-20 in 1x PBS, pH 7.6) for 4x 15 min and developed with Nitro blue tetrazolium chloride/5-broma-4-chloro-3-indoyl phosphate substrate (NBT/BCIP substrate; Roche). Chimaera expression was compared by measuring the density of the bands detected on anti-L1 western blots using GeneTools (SYNGENE).

2.2.9 Chimaera quantification by capture ELISA

The L1 chimaeras extracted from *N. benthamiana* were quantified by capture ELISA using a modified polyvinyl alcohol (PVA)-blocking ELISA method (Studentsov *et al.*, 2002). Briefly, a 96-well Maxisorp microtitre plate was coated with 1:2000 mouse anti HPV-16 L1 MAb (either CamVir1 or H16.J4) overnight at 4°C and blocked with PVA. Plant extract was added to the wells and incubated for 1 hr at 37°C. This was followed by a washing step and the addition of rabbit anti-HPV-16 polyclonal serum (1:1000). The plate was incubated overnight at 4°C and HPV-16 L1 protein was detected with swine anti-rabbit horseradish peroxidase (HRP) conjugate (1:5000; DAKO) and 1.2-phenylenediamine dihydrochloride substrate (OPD; DAKO; Denmark).

The commercial HPV vaccine Cervarix was used as a positive ELISA control and as a HPV-16 L1 VLP standard. Each sample was analysed in triplicate and quantified using the Cervarix standard curve. The amount of chimaera

protein present in each sample (mg) was expressed as chimaera per kilogram of plant tissue (mg/kg).

Total soluble protein (TSP) for each crude leaf extract was determined using the Lowry protein assay (Biorad DC Protein Assay; Microplate Assay Protocol) as per the manufacturer's instructions using a Bovine plasma gamma globin IgG protein standard (Bio-Rad). The relative chimaera yield was calculated where the ELISA-quantified chimaera protein (mg) was expressed as a percentage of TSP, in order to account for differences in leaf tissue mass and protein extraction efficiency.

2.2.10 Statistical analysis of chimaera expression yields

Statistical differences in chimaera expression using the different plant expression vectors were determined using ANOVA and the Fischer LSD Post Hoc test. Differences were reported at statistically significant at $p < 0.01$.

2.2.11 Chimaera assembly

Assembly of the HPV proteins into higher-order immunogenic structures was assessed using a H16.J4 and H16.V5 capture ELISA as described above. The H16.J4 MAb binds to a L1 linear epitope comprising of aa 261-280 (Christensen *et al*., 1996) and thus gives the total HPV protein present in the plant extract. H16.V5 binds to a conformational L1 epitope (Christensen *et al*., 1996, 2001) containing essential aa 260-290 and specifically binding L1 residues Phe-50, Ala-266, and Ser-282 (White *et* al., 1999), thus it was used for the detection of assembled HPV protein. In order to compare the assembly of chimaeras expressed using different vectors, the amount of assembled HPV protein was expressed as a percentage of the total HPV protein.

2.3 Results

2.3.1 Verification of L1 chimaera clones

The L1 chimaeras (Table 1) were successfully cloned into the pTRAkc-rbcs1-cTP and pRIC3 plant expression vectors and transformed into *E. coli* and *Agrobacterium* GV3101.

The pTRAkc-rbcs1-cTP recombinant clones were screened by colony PCR using pTRAc-specific primers binding upstream and downstream of the MCS (Figure 3A), or chimaera-specific primers binding to different L2 or E7 epitopes (Figure 3B). All chimaeras produced fragments of the expected size (Table 3), with 1.7 kb fragments for the constructs in Figure 3A and 0.2 - 0.6 kb fragments for the individual chimaeras in Figure 3B.

Clones were further verified by restriction enzyme (RE) digestion using *Eco*RI and *Xho*I RE sites which flank the chimaera gene insert. As expected, all chimaeras contained a 1.5 kb gene insert (Figure 4). Clones were sequenced and individual chimaeras were confirmed using DNAMAN multiple sequence alignment software (data not shown).

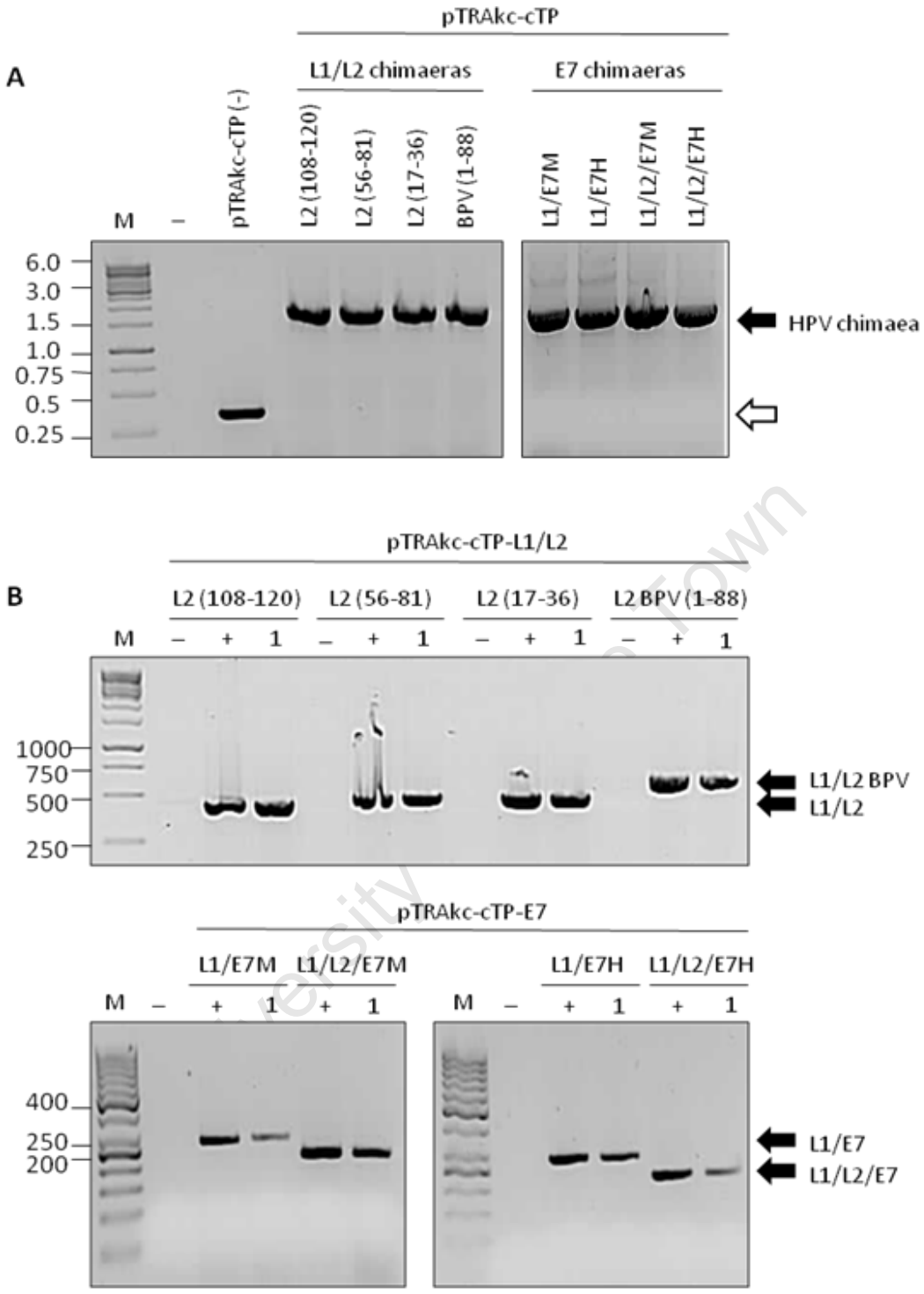

Figure 3: L1 chimaeras cloned into pTRAc-rbcs1-cTP were identified by colony PCR using A) vector-specific primers and B) L2 or E7 epitope-specific primers. Labels: M = DNA marker with size in kb indicated on the left. –ve control = no template water control with the appropriate primer set. pTRAkc-cTP (-) = non-recombinant vector control. +ve control = parental pGA4 chimaera clones. 1 = Recombinant clone tested. Black arrows represent the L1 chimaera PCR products. White arrow represents the ~0.2 kb PCR product for the non-recombinant vector.

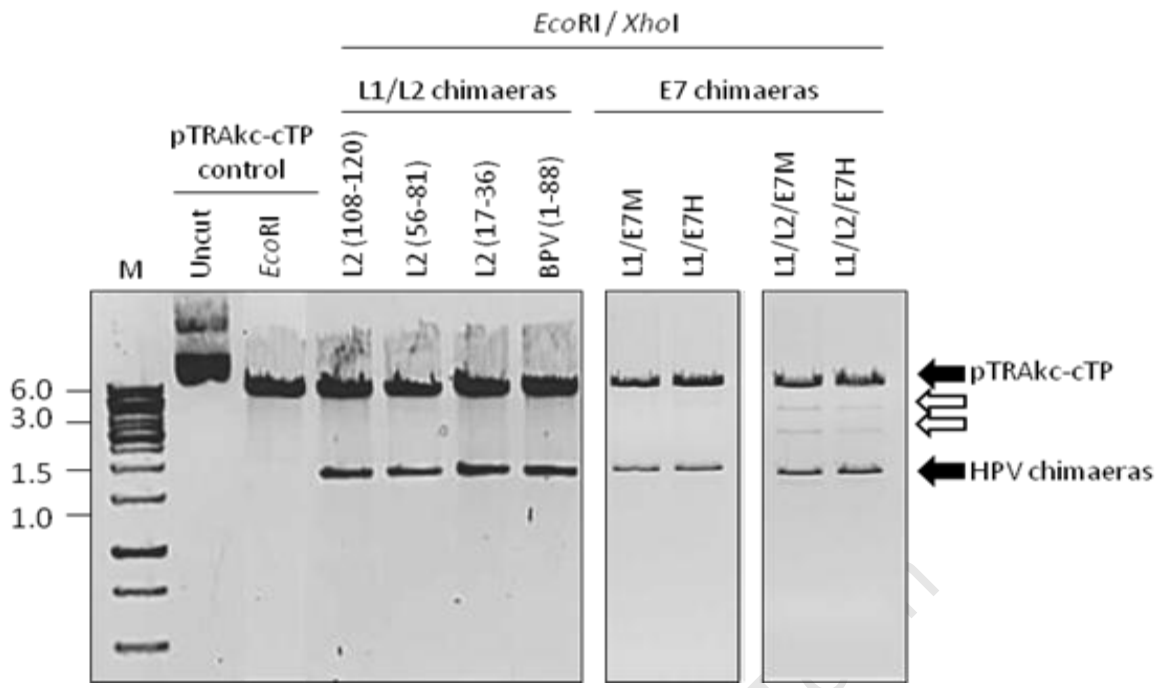

Figure 4: L1 chimaera recombinant clones were verified by *Eco*RI / *Xho*I restriction enzyme digests. M = DNA marker with size in kb indicated on the left. pTRAkc-cTP control = non-recombinant vector. Black arrows indicate the ~1.5 kb L1 chimaera insert and the ~7.7 kb linearized vector. White arrows represent additional bands caused by *Eco*RI star activity.

The pRIC3 recombinant clones were similarly verified by colony PCR using the chimaera epitope-specific primers and *Hind*III / *Xho*I restriction enzyme digestion (Figure 5). All chimaeras produced the 0.2–0.6 kb chimaera-specific PCR bands (Figure 5A) described in Table 3 and the 1.5 kb gene fragment in the RE digests (Figure 5B). Thus all the HPV chimaeras were successfully subcloned into the pTRAkc-rbcs1-cTP and pRIC3 plant expression vectors.

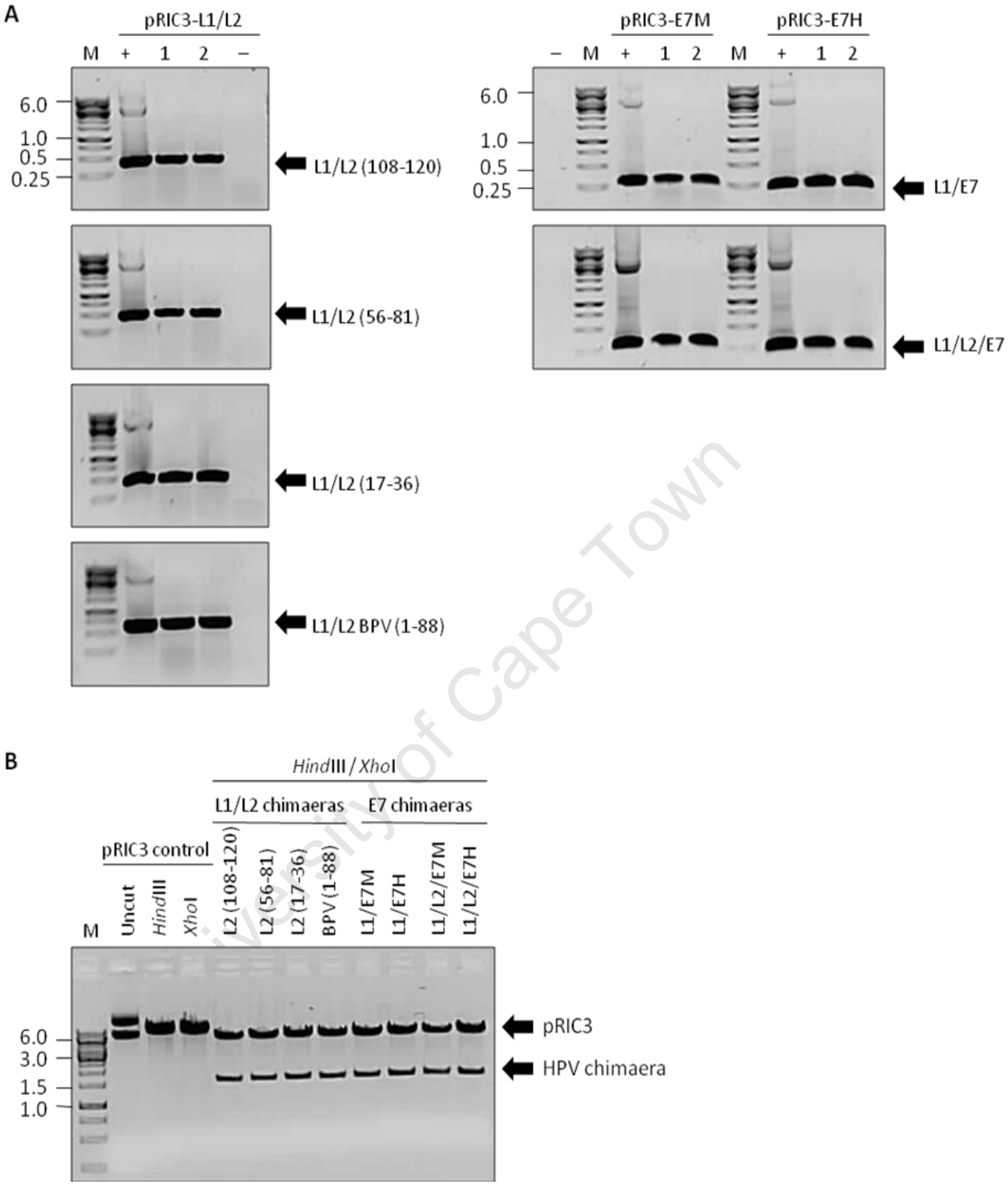

Figure 5: L1 chimaeras cloned into pRIC3 were identified by colony PCR and verified by RE digestion. A) Colony PCR using L2 or E7 epitope-specific primers, black arrows represent the L2 or E7-epitope specific PCR products. B) *Hind*III / *Xho*I RE digests, black arrows represent the ~1.5 kb L1 chimaera gene insert and the ~6 kb linearized pRIC3 vector. Labels: M = DNA marker with size in kb indicated on the left. –ve control = no template water control with the appropriate primer set. +ve control = parental pGA4-HPV chimaera clones. 1 and 2 = Recombinant clones tested.

2.3.2 Optimisation of L1 chimaera expression in *N. benthamiana*

2.3.2.1 Co-expression with the NSs silencing suppressor

Chloroplast-targeted HPV-16 L1/L2 expression in *N. bethamiana* was examined in a 1-9 day post-infiltration (dpi) time trial. Chimaeras were expressed either with (+) or without (-) the NSs silencing suppressor protein to examine its effects on chimaera expression. Expression was analysed by western blotting using the anti-L1 MAb CamVir1. All the L1/L2 chimaeras were detected, with the predicted ~56 kDa L1 band (Figure 6), although L1/L2(108-120) runs higher than the other chimaeras.

All chimaeras showed a prolonged increase in expression when co-infiltrated with the silencing suppressor protein NSs (Figure 6A-D), suggesting it was effective in preventing post-transcriptional gene silencing and enhancing protein accumulation in plants. ELISA detection using the linear-epitope specific MAb H16.J4 confirmed the results, with up to a 16-fold increase in L1/L2 yields (data not shown). Chimaera expression without NSs was detected 1-3 dpi and peaks 3-5 dpi, while chimaeras co-expressed with NSs was detected at 3 dpi and expression peaked at 5-7 dpi. There was a small decrease in expression between 5-9 dpi, suggesting there is a slow decline in expression levels (ELISA results, data not shown). As a result, all chimaeras were co-expressed with NSs in further experiments.

Several high molecular bands were detected for the L1/L2(17-36) chimaera, suggesting the chloroplast signal sequence (cTP) may not have been cleaved or the chimaera may have been glycosylated. However, L1/L2(17-36) analysed on subsequent western blots did not display these high molecular weight bands, suggesting the protein was partially denatured in Figure 6C.

The L1/L2 chimaera containing the BPV L2 aa 1-88 epitope had low expression levels in comparison to the chimaeras containing HPV-16 L2 epitopes. The bands on the L1/L2 BPV (1-88) western blots were only visible after 16 hours of development (Figure 6D), in comparison to the 15 min

development time required for the other chimaeras (Figure 6A-C). ELISA quantification estimated L1/L2 BPV(1-88) achieved maximum yields of 40 mg/kg plant tissue, while high expression yields of 1000 - 4600 mg/kg were estimated for the other L1/L2 chimaeras (data not shown). In addition, the L1/L2 BPV(1-88) plant extract contained a characteristic ~45 kDa band (Figure 6D) associated with L1 degradation, suggesting L1/L2 BPV(1-88) is unstable in this expression system. These results were confirmed by several L1/L2 BPV(1-88) western blots from different time trials (data not shown).

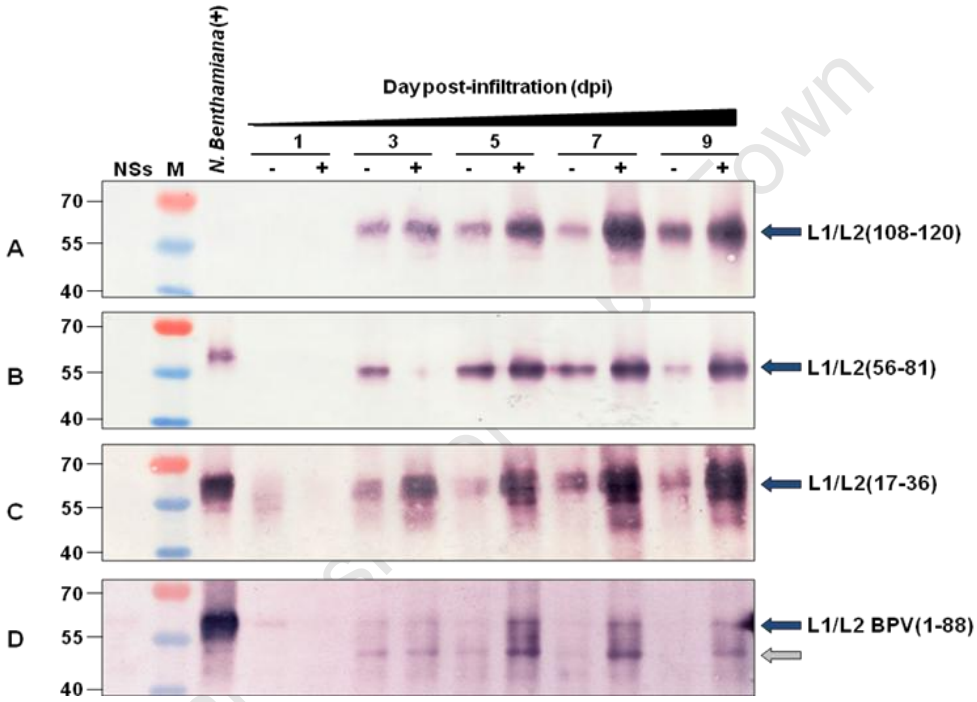

Figure 6: Chloroplast-targeted L1/L2 chimaera expression time trial 1-9 days post-infiltration (dpi) in *N. benthamiana*, either with (+) or without (-) the NSs silencing suppressor. The L1/L2 chimaeras A) L1/L2(108-120), B) L1/L2(56-81), C) L1/L2(17-36) and D) L1/L2 BPV (1-88) in crude leaf extracts were detected by CamVir1 western blot analysis. Equal volumes were loaded and blots were developed for 15 minutes (A-C) and for 16 hours (D). M = protein marker with the size in kDa indicated on the left. NSs negative control = pBIN-NSs infiltrated crude plant extract (5 dpi). Positive controls: *N. benthamiana* (+) = plant-derived HPV-16 L1. The black arrows indicate the position of the L1/L2 chimaeras (~56 kDa) and the grey arrow indicates degraded protein.

2.3.2.2 Effect of chloroplast targeting on L1/L2 chimaera yield

Targeting of HPV proteins to the chloroplast can significantly improve plant expression yields (Maclean *et al*., 2007). To determine the importance of

chloroplast-targeting, the pTRAc (cytoplasmic-targeting) and the pTRAkc-rbcs1-cTP (chloroplast-targeting) L1/L2 chimaera constructs were co-infiltrated with pBIN-NSs in *N. benthamiana* in a 3-9 dpi time trial (Figure 7). The L1/L2 BPV(1-88) chimaera was not included in this study, as it shows very low expression in *N. bethamiana* when compared to the other L1/L2 chimaeras (Figure 6).

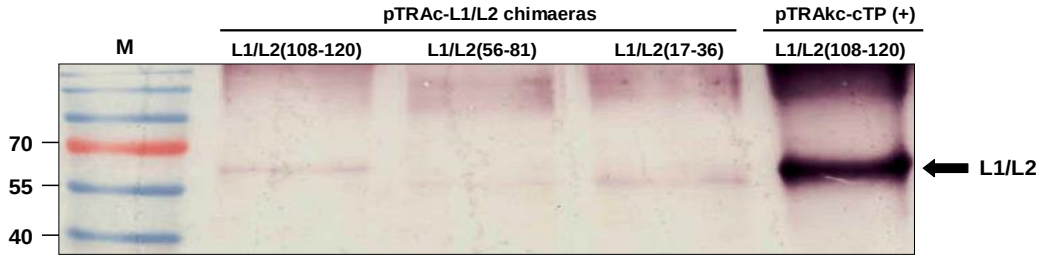

Figure 7: Expression of cytoplasm-targeted L1/L2 chimaeras in *N. benthamiana* when co-infiltrated with the pTRAc chimaera constructs and pBIN-NSs. L1/L2(108-120), L1/L2(56-81) and L1/L2(17-36) chimaeras were detected with H16.J4 in crude *N. benthamiana* leaf extracts 3 dpi. M = protein marker with the size in kDa indicated on the left. pTRAkc-cTP (+) = chloroplast-targeted pTRAkc-rbcs1-cTP L1/L2(108-120) with pBIN-NSs at 3 dpi (positive control). Equal volumes were loaded but pTRAkc-rbcs1-cTP L1/L2(108-120) extract was diluted 3-fold to prevent the over-exposure of bands. The arrow indicates the position of the L1/L2 chimaeras (~56 kDa).

Western blots and ELISA data consistently demonstrated low expression for the cytoplasm-targeted L1/L2 chimaeras, with maximum expression of chimaeras 3 dpi and yields of 20-45 mg/kg plant tissue (data not shown). An example of a western blot is shown in Figure 7. Expression of cytoplasm-targeted L1/L2(108-120), L1/L2(56-81) and L1/L2(17-36) was weakly detected in comparison to the chloroplast-targeted L1/L2(108-120) chimaera diluted 3x prior to loading and included as a positive control. Comparison of chimaera yields indicates that L1/L2 chimaera expression was increased 40 - 80 fold when targeted to the chloroplast. Taking these results into consideration, further chimaera expression studies were done using the pTRAkc-rbcs1-cTP vector.

2.3.2.3 Transient expression of the E7 chimaeras

Chloroplast-targeted L1/E7 and L1/L2/E7 chimaeras, co-expressed with NSs, were examined in a 1-9 day post-infiltration (dpi) time trial. All the E7 chimaeras were successfully expressed in *N. benthamiana* (Figure 8), although yields were low. Maximum yields of 10-40 mg/kg plant tissue was obtained 3-5 dpi and ELISA results demonstrated both the L1/E7 chimaeras are expressed to higher levels than the corresponding L1/L2/E7 chimaeras (data not shown), suggesting the insertion of the second epitope negatively affects chimaera expression.

Two bands were detected for the L1/E7 chimaeras: the ~56 kDa band and an unidentified ~65 kDa band (Figure 8A). Repeated L1/E7 time trial analyses demonstrated only the 56 kDa L1 band, suggesting the L1/E7 chimaera was not denatured sufficiently, as described for L1/L2(17-36).

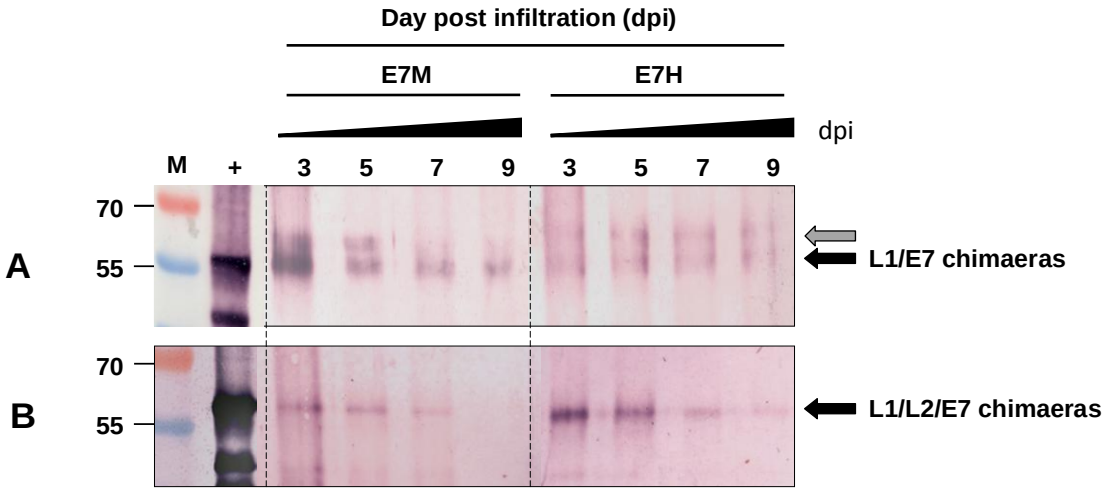

Figure 8: Chloroplast-targeted E7 chimaera expression time trial 3-9 days post co-infiltration with the *Agrobacterium* chimaera strain and pBIN-NSs in *N. benthamiana*. CamVir1 western blot detection of A) L1/E7 and B) L1/L2/E7chimaeras in crude *N. benthamiana* leaf extracts 3-9 dpi. M = protein marker with the size in kDa indicated on the left. Positive control (+) = crude insect-cell produced L1/E7M or L1/L2/E7M. The black arrow indicates the position of the E7 chimaeras (~56 kDa). The grey arrow is caused by insufficient denaturation or glycosylation of the L1/E7 chimaeras.

2.3.2.4 Optimisation using the self-replicative pRIC3 plant expression vector

In an attempt to improve chimaera yields, particularly for the low-expressing L1/L2 BPV(1-88) and E7 chimaeras, the plant expression vector pRIC3 (self-replicative, cytoplasm-targeting vector) was compared to pTRAkc-rbcs1-cTP (non-replicative chloroplast-targeting vector) in a 3-9 dpi time trial in the presence of NSs (Figure 9). The pRIC vector has been shown to improve yields for several proteins, including HPV-16 L1 (Regnard *et al*., 2009).

Western blot analysis of L1/L2 expression is shown in Figure 9A. Maximum chimaera yields for both vectors were obtained 3-5 dpi. The three L1/L2 chimaeras containing the HPV-16 L2 epitopes aa 108-120, 56-81 and 17-36 were better expressed using the chloroplast-targeting pTRAkc-rbcs-cTP vector compared to the self-replicative pRIC3 vector. L1/L2 BPV(1-88) was not highly expressed for either vector and degradation was visible for both constructs. Western blot detection of L1/E7 and L1/L2/E7 expression showed similar expression profiles for both vectors, with maximum expression 3-5 dpi (data not shown).

ELISA quantification shows the self-replicative pRIC3 vector did not improve expression yields for majority of the chimaeras. Yields were up to 3-fold higher using the pTRAkc-rbcs1-cTP vector, suggesting chloroplast-targeting is more effective in the high-expression of chimaeras than the pRIC3 vector, which ultimately targets the expressed protein to the cytoplasm. The only exception was low-expressing L1/L2/E7M, which gave similar yields using either vector (Figure 9B). The L1/L2 BPV(1-88) expression levels were similar to the NSs negative control, suggesting plants are not a viable system for the production of L1/L2 BPV(1-88) and the expression of L1/L2 BPV(1-88) was not pursued further.

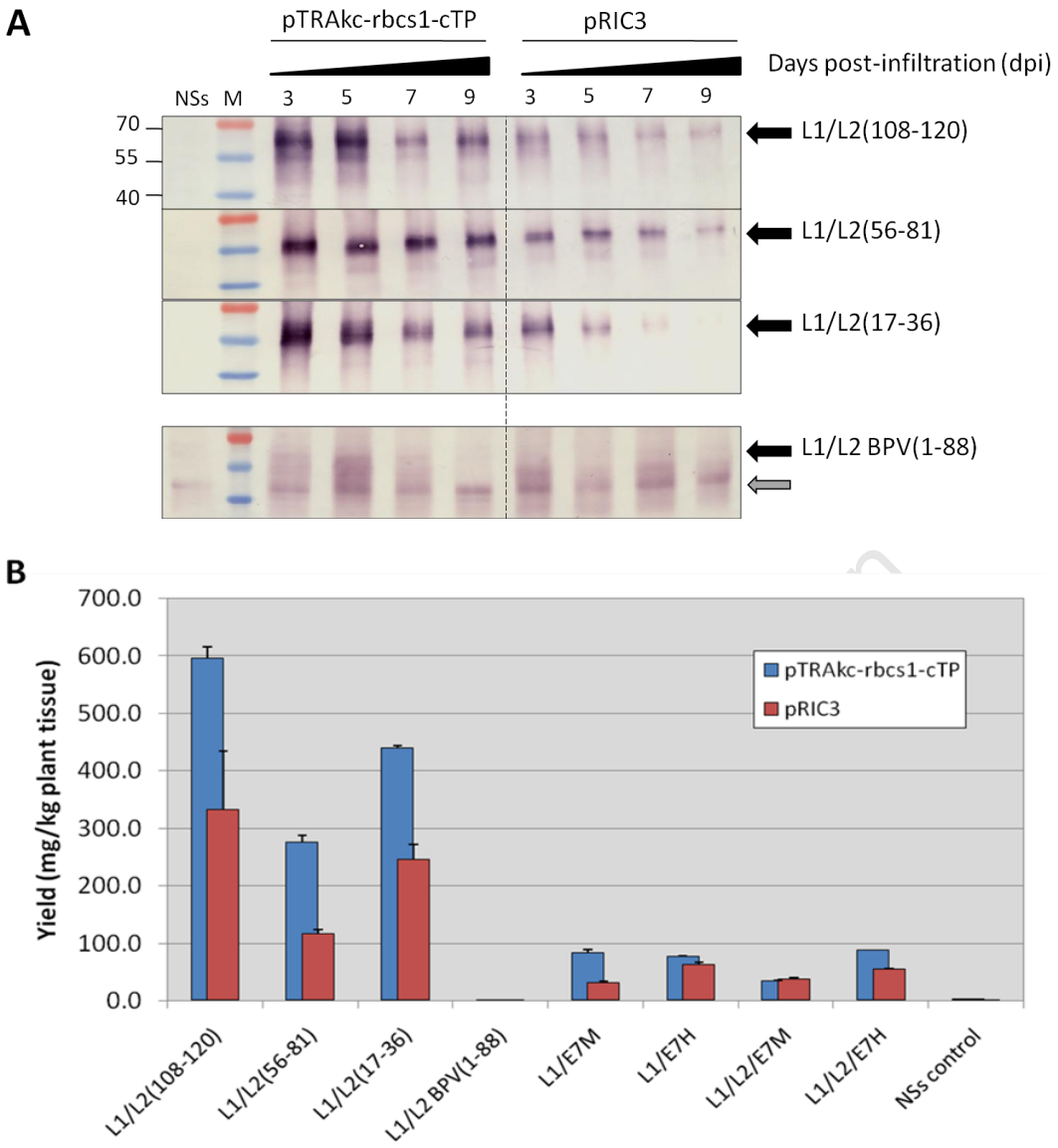

Figure 9: Comparative expression time trial 3-9 days post-infiltration (dpi) of *N. benthamiana* using the vectors pTRAc-rbcs1-cTP and pRIC3. A) CamVir1 western blot analysis of the L1/L2 chimaeras. Similar volumes were loaded for all samples, although the high-expressing L1/L2 chimaera samples were diluted 8-fold compared to the low-expressing L1/L2 BPV(1-88). M = protein marker with the size in kDa indicated on the left. NSs negative control = pBIN-NSs infiltrated crude leaf extract 5 dpi. The black arrow indicates the HPV chimaeras (~56 kDa). The grey arrow indicates degraded chimaeras. B) Capture ELISA quantification of all the HPV chimaeras using CamVir1. The highest yield obtained for each chimaera, occurring either 3 or 5 dpi, is shown for the two vectors. The error bars indicate the standard deviation.

The results from the expression optimization using the pTRAkc-rbcs1-cTP and pRIC3 vectors are summarised in Table 4. The L1/L2(108-120), L1/L2(56-81) and L1/L2(17-36) were highly-expressed, with yields 3-20 fold higher than the other chimaeras. The parameters shown to maximise expression in the

preliminary time trials are: co-expression with NSs (Figure 6), extraction 5 dpi (Table 4) and use of the pTRAkc-rbcs-cTP vector to target the expressed L1/L2 protein to the chloroplast (Figure 7 and 9; Table 4).

Table 4: Summary of L1 chimaera expression and optimization

	Maximum chimaera expression				
Chimaera	**Extraction (dpi)**	**Vector**	**Yield (mg/kg)**	**Yield (%TSP)**	**Fold increase (pTRAkc-rbcs1-cTP vs. pRIC3)**
L1/L2(108-120)	5	pTRAkc-rbcs1-cTP	600	3.7	1.8
L1/L2(56-81)	5	pTRAkc-rbcs1-cTP	280	1.7	2.4
L1/L2(17-36)	5	pTRAkc-rbcs1-cTP	440	2.9	1.8
L1/L2 BPV(1-88)	5	pTRAkc-rbcs1-cTP	-	-	-
L1/E7M	3-5	pTRAkc-rbcs1-cTP	80	0.6	2.8
L1/E7H	3	pTRAkc-rbcs1-cTP	80	0.4	1.2
L1/L2/E7M	3-5	pRIC3	30	0.3	0.9
L1/L2/E7H	3-5	pTRAkc-rbcs1-cTP	90	0.8	1.6

2.3.3 Comparative vector expression of L1/L2 chimaeras

Three high-expressing L1/L2 chimaeras were chosen as vaccine antigens for the mouse immunogenicity studies: L1/L2(108-120), L1/L2(56-81) and L1/L2(17-36). A final expression study including three biological repeats was performed to confirm the L1/L2 results and obtain statistically valid data. All three vectors (pTRAc, pTRAkc-rbcs1-cTP and pRIC3) were directly compared for expression of each of the L1/L2 vaccine antigens, HPV-16 L1 was included as a positive control (pTRAc and pTRAkc-rbcs1-cTP constructs were available) and NSs-infiltrated plants served as the negative control. Chimaeras were co-expressed with NSs and extracted 5 dpi.

2.3.3.1 Effect of expression on plants

Examination of the infiltrated leaves prior to extraction 5 dpi (Figure 10) suggested that the self-replicative pRIC3 vector had adverse effects on the health of the plant. Leaves infiltrated with pRIC3 were yellow/brown in colour and necrosis of the leaf tissue was visible in the infiltrated areas. This was observed to a lesser degree in the pTRAc leaves, which also targeted chimaeras to the plant cytoplasm. The pTRAkc-rbcs-cTP leaves appeared to

be the healthiest, resembling the leaves of the NSs-infiltrated negative control and the uninfiltrated leaves, suggesting accumulation of the chimaras in the chloroplast has less of an impact on plant health. Infiltration appears to have no observable effect on plant health, as the NSs-infiltrated leaf looked similar to the uninfiltrated leaf (excluding the syringe injection markings). These results were consistently observed for all the time trials.

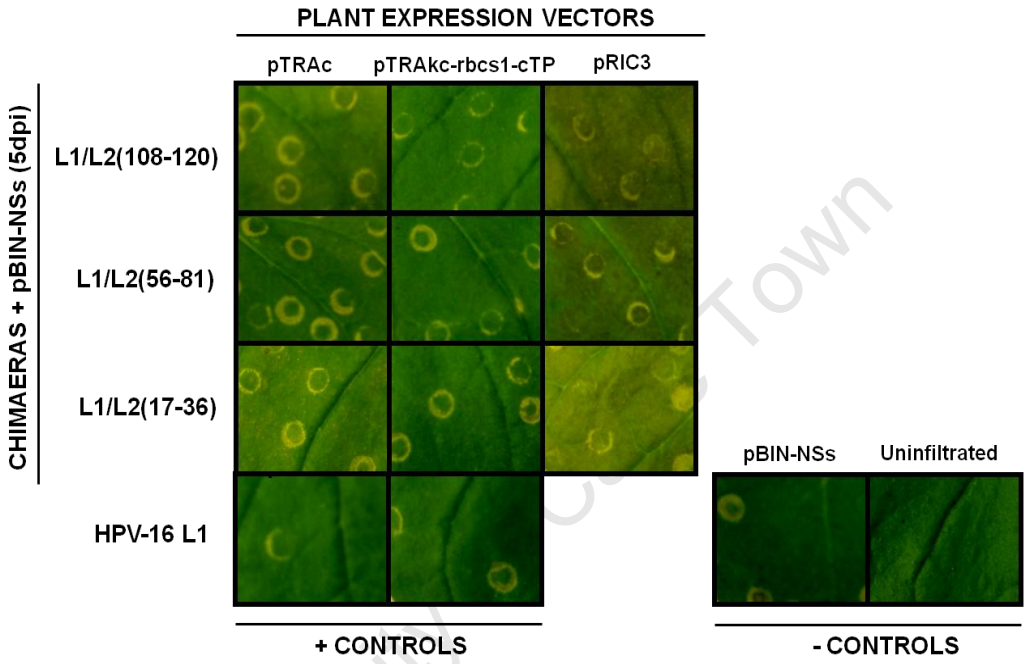

Figure 10: Photographs of infiltrated leaves prior to chimaera extraction at 5 days post-infiltration. Three plant expression vectors were used to express the L1/L2 chimaera vaccine antigens: pTRAc, pTRAkc-rbcs1-cTP and pRIC3. HPV-16 L1 was expressed as a positive expression control and NSs-infiltrated plants served as the negative expression control. The uninfiltrated leaf was used as a health indicator for the observable effects of infiltration and protein expression using the vectors.

2.3.3.2 Western blot detection of the HPV proteins

HPV protein was detected by anti-L1 western blotting (Figure 11). The NSs-infiltrated plant extract (negative control) was not detected and plant-derived HPV-16 L1 (positive control) was detected using the chloroplast-targeting vector. Expression using the different vectors was directly compared with pTRAkc-rbcs1-cTP consistently giving the highest expression yields, followed by pRIC3, and then pTRAc.

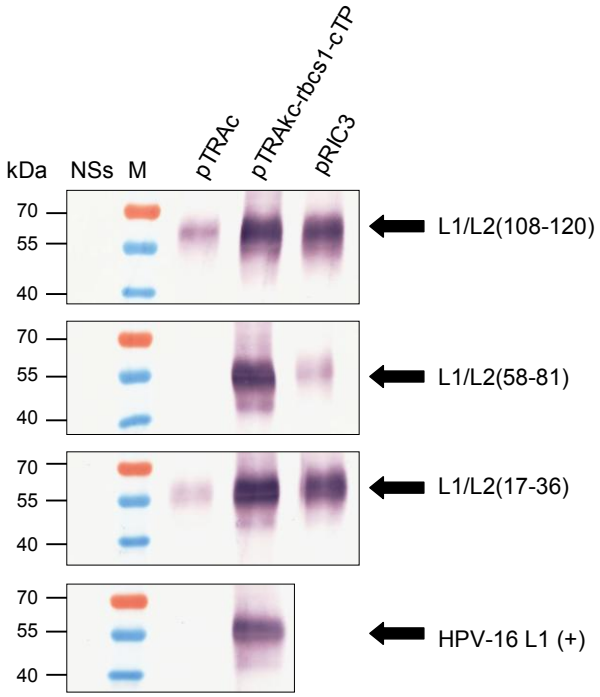

Figure 11: Western blot of the L1/L2 chimaeras expressed using 3 plant expression vectors: pTRAc, pTRAkc-rbcs1-cTP and pRIC3. Chimaras were co-expressed with NSs, extracted 5 dpi and detected with CamVir1. HPV-16 L1 was expressed as a positive expression control for pTRAc and pTRAkc-rbcs1-cTP (pRIC3 construct not available) and the negative expression control was NSs-infiltrated plants. Equal volumes of sample were loaded. M = protein marker with the size of the protein indictated in kDa on the left. The black arrows indicate the the L1/L2 chimaeras or HPV-16 L1 (~56 kDa).

2.3.3.3 ELISA quantification of the HPV proteins

Capture ELISA was used to quantify the HPV chimaeras using CamVir1. The L1/L2 chimaera and HPV-16 L1 yields are shown in Figure 12. Statistical differences in chimaera expression using the 3 plant expression vectors were determined using ANOVA and the Fischer LSD Post Hoc test. Differences were reported at statistically significant at $p < 0.01$.

Chloroplast-targeted expression of the L1/L2 chimaeras and HPV-16 L1 using pTRAkc-rbcs1-cTP gave significantly higher yields than the NSs-infiltrated negative control (p = 0.000 - 0.002), and the cytoplasm-targeting pTRAc vector (p = 0.000 - 0.004). In addition, pTRAkc-rbcs1-cTP significantly improved L1/L2(56-81) expression compared to pRIC3 (p = 0.006). The pRIC3 vector did not statistically improve expression of any of the chimaeras compared to pTRAc.

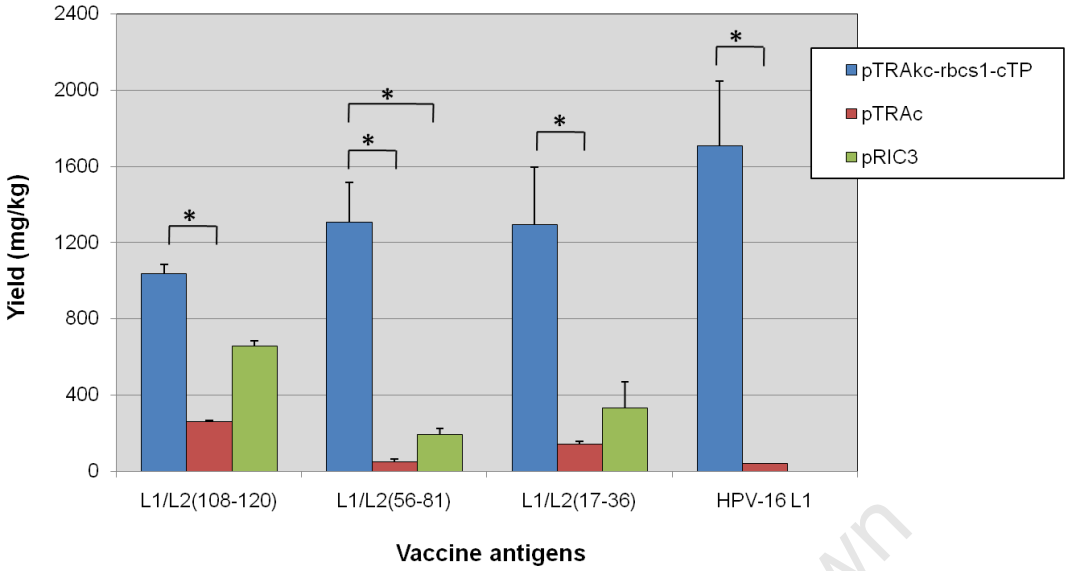

Figure 12: Comparison of the L1/L2 chimaeras expressed using 3 plant expression vectors: pTRAc, pTRAkc-rbcs1-cTP and pRIC3. Proteins were co-expressed with the NSs, extracted 5 days post-infiltration and HPV protein yield was determined by capture ELISA using CamVir1. HPV-16 L1 was expressed as a positive expression control. Statistical analysis by ANOVA and the Fisher LSD Post-Hoc test showed significant differences in chimaera yields and yields that are significantly different at p = 0.01 are denoted with a star. The error bars indicate the standard deviation.

In comparison to the optimization experiments (Figure 6-9, Table 4), the comparative time trial demonstrated similar trends in chimaera expression. The chloroplast-targeted L1/L2 chimaeras gave the highest yields (1040 - 1310 mg/kg; 2 - 3% TSP), improving chimaera expression by up to 28-fold in comparison to the cytoplasm-targeting vector pTRAc (50 – 260 mg/kg; <1% TSP) and up to 7-fold in comparison to the self-replicative vector pRIC3 (190 - 660 mg/kg; <1% TSP).

Cytoplasm-targeted chimaera yields were improved up to 4-fold using the self-replicative vector pRIC3 in comparison to the non-replicative pTRAc vector. This suggests self-replication of the vector improves chimaera expression, although targeting to the chloroplast appears to be a superior strategy to increase chimaera expression in plants.

Although chloroplast-targeted HPV-16 L1 demonstrated higher average yields (1710 mg/kg, 4% TSP), the differences between the L1/L2 chimaeras and L1

were not statistically significant, indicating the L2 epitope substitutions do not appear to affect the expression and accumulation of recombinant protein in chloroplasts. However, western blotting (Figure 7 and 11) and ELISA expression data (Figure 12) consistently revealed higher levels of cytoplasm-localised L1/L2(108-120) and L1/L2(17-36) than L1/L2(56-81), suggesting L1/L2(108-120) and L1/L2(17-36) chimaeras with shorter L2 sequence replacements (13 and 20 aa respectively) may be better expressed and have a greater stability than L1/L2(56-81) with a 26 aa sequence replacement.

2.3.3.4 Assembly of the HPV proteins

Chimaera assembly into higher-order structures such as capsomeres or VLPs was assessed using H16.J4 (linear epitope-specific MAb) and H16.V5 (conformational epitope-specific MAb) capture ELISA. The amount of V5-detected conformational HPV protein was expressed as a percentage of the J4-detected total HPV protein for each of the vector constructs (Figure 13).

A low percentage of the expressed chimaeras assembled into H16.V5-detected higher-order structures. The NSs plant extract, used as a negative control, did not bind H16.J4 or H16.V5 MAb (data not shown). The low-expressing pTRAc chimaeras appear to have the highest proportion of assembled protein (11-18%), followed by the high-expressing pTRAkc-rbcs1-cTP chimaeras (5-9%). The pRIC3 chimaeras did not contain a high percentage of assembled protein (< 2%). Although the pTRAkc-rbcs1-cTP chimaeras did not contain the highest percentage of assembled protein, higher expression yields results in up to 40x and 4x more assembled protein than pTRAc and pRIC3 respectively. This provides further evidence that pTRAkc-rbcs1-cTP is the best vector to use for the production of immunogenic L1/L2 chimaeras.

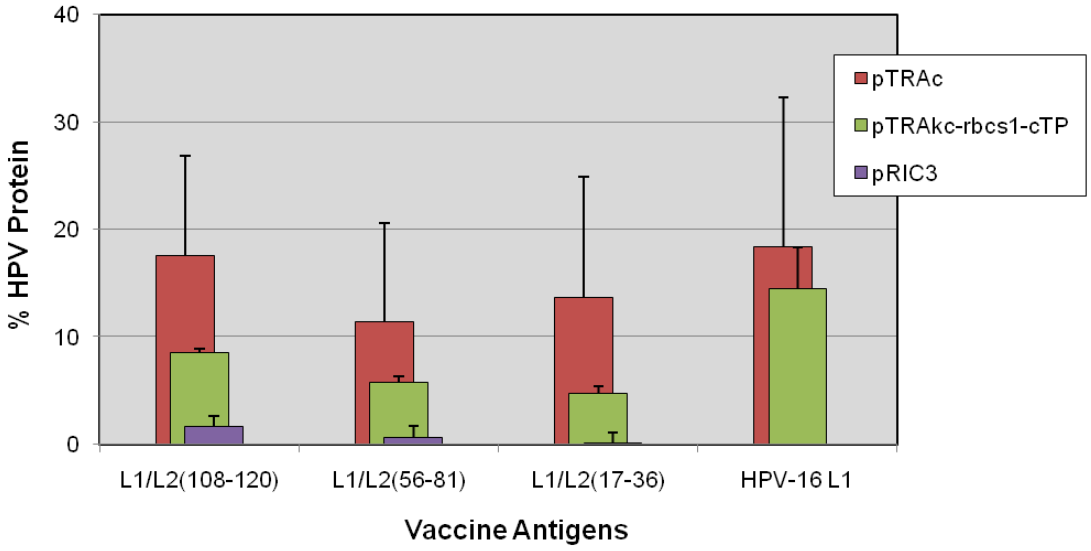

Figure 13: Assembly of L1/L2 chimaeras expressed using 3 different plant expression vectors: pTRAc, pTRAkc-rbcs1-cTP and pRIC3. Proteins were co-expressed with the NSs silencing suppressor and extracted 5 dpi. Chimaeras assembled into higher-ordered structures such as capsomeres or VLPs (detected by conformational-specific H16.V5 MAb) is expressed as a percentage of the total chimaera protein (detected by the linear-specific H16.J4 MAb). HPV-16 L1 was expressed as a positive expression control and the negative expression control was NSs-infiltrated plants. The error bars indicate the standard deviation.

2.4 Discussion

2.4.1 Optimisation of L1 chimaera transient expression in plants

All the L1 chimaeras were successfully expressed in plants using an *Agrobacterium*-mediated transient system (Figure 6-9). Several methods were used to optimize chimaera expression in plants; including use of a NSs silencing suppressor, use of an agroinfiltration-delivered self-replicative viral vector and targeting of the expressed protein to the chloroplast.

2.4.1.1 Co-expression with the NSs silencing suppressor

Agrobacterium-mediated transient expression typically peaks 60-72 hours (~3 days) post-infiltration and then declines rapidly as a result of triggering post-transcriptional gene silencing (PTGS) in the host plant (Voinnet, 2001). PTGS is an adaptive anti-viral plant defense mechanism, where foreign RNA molecules are recognized and degraded in a sequence-specific manner (Meins, 2000; Sijen and Kooter, 2000). As a counter-defensive strategy, many

plant viruses have evolved proteins that suppress various steps of the mechanism (Voinnet, 2001). Although PTGS responses reduce transgene mRNA accumulation in the plant cytoplasm and limit the efficiency of *Agrobacterium*-mediated transient expression (de Carvalho *et al*., 1992; Van Blokland *et al*., 1994), co-expression of proteins with viral silencing suppressors has been shown to repress PTGS responses and allow high level transient expression, resulting in higher yields (50-fold in some instances) and prolonged expression of the transgene (Voinnet *et al.,* 2003).

Co-infiltration with the tomato spotted wilt virus (TSWV) silencing suppressor NSs suppresses PTGS and increases transient expression (Takeda *et al*., 2002). This effect was similarly observed in the transient expression of the L1/L2 chimaeras (Figure 6). Chimaeras typically displayed maximum expression levels 3-5 dpi without the presence of viral silencing suppressors. However, co-expression with NSs displayed a prolonged increase in the expression of the chimaeras, whereby expression levels were increased by up to 16-fold and peaked 5-7 dpi.

2.4.1.2 The use of a self-replicative BeYDV vector

Cytoplasmic HPV-16 L1 yields have been improved by 50% using a self-replicative pRIC vector (Regnard *et al*., 2010). As a result, a third-generation pRIC3 vector was examined as a potential strategy to improve chimaera yields. Three L1/L2 chimaeras were examined: L1/L2(108-120), L1/L2(56-81) and L1/L2(17-36). All chimaeras demonstrated higher expression levels using pRIC3 (self-replicative vector), in comparison to pTRAc (non-replicative vector), suggesting transgene amplification improves L1/L2 yields in the plant cytoplasm (Figure 11 and 12). However, chloroplast-targeting was more effective in the high-level accumulation of L1/L2 chimaeras (Figure 12) and visible necrosis of the leaf tissue was observed in pRIC3-infiltrated leaves, suggesting the self-replication of the vector negatively affects plant health (Figure 10).

2.4.1.3 Chloroplast-targeting of L1 chimaeras

L1 chimaeras were targeted to the chloroplast using the pTRAkc-rbcs1-cTP vector. The chloroplast transit peptide (cTP) is fused to the expressed chimaera and is cleaved by the chloroplast stromal processing peptidase (SPP) upon entry into the organelle (Robinson and Ellis, 1984). There are several factors responsible for the high level accumulation of protein in chloroplasts: (a) protection from cellular proteases, (b) different protein hydrolyzing machinery in the plastids and (c) protective plasmid-specific chaperones which assist in the correct folding of L1 and thus improve protein stability (Fernández-San Millán *et al*., 2008). Previous studies have shown that targeting HPV-16 L1 to the chloroplast produces higher yields than targeting to the cytoplasm or ER (Maclean *et al*., 2007). In this study, chloroplast-targeting was highly effective and increased L1/L2 chimaera yields by 40 to 80-fold in comparison to chimaeras targeted to the cytoplasm (Figure 7 and 11).

The chloroplast-targeted chimaeras detected in the anti-L1 western blots produced bands of ~56 kDa (Figure 6-9), suggesting the signal peptide was effectively removed from the accumulated protein. Although L1/L2(108-120) runs higher than the other chimaeras on the western blot (Figure 6 and 8), this phenomenon is not caused by insufficient cleavage of the signal peptide, as the cytoplasm-localised L1/L2(108-120) expressed with pTRAc (Figure 7), and insect cell-expressed L1/L2(108-120) analysed in parallel (data not shown), showed a similar banding pattern.

Higher molecular weight bands of ~65 kDa were detected for L1/L2(17-36) and the L1/E7 chimaeras (Figure 6 and 8), possibly as a result of glycosylation or insufficient denaturation of chimaeras. A glycosylated form of HPV-16 L1 produced in baby hamster kidney cells (BHK) was described by McLean *et al*. (1990), whereby CamVir1 detected 2 bands for L1: the 56 kDa L1 major band and a minor band of ~64 kDa. The additional band was subsequently removed from cell lysates when infected in the presence of the N-glycosylation inhibitor tunicamycin. Although plants do contain glycosylation pathways (Rybicki, 2009), subsequent western blots displayed a single

~56 kDa band, suggesting the L1/L2(17-36) and L1/E7 chimaeras were partially denatured in initial experiments rather than glycosylated (L1/L2: Figure 9A and 11, L1/E7: data not shown).

2.4.1.4 Direct comparison of plant expression vectors

Two strategies have increased plant-expressed L1 yields to a maximum of 530 - 550 mg/kg: targeting the protein to the chloroplast (Maclean *et al*., 2007) or the use of an agroinfiltration-delivered self-replicative BeYDV-derived expression vector (Regnard *et al*., 2010). This was the first study which directly compared these strategies using the L1-based chimaeras. Chimaera expression levels using the plant expression vector pRIC3 (self-replicative, cytoplasm-targeting vector) was directly compared to pTRAkc-rbcs1-cTP (non-replicative, chloroplast-targeting vector) in the presence of NSs (Figure 9). Expression using the pTRAc (non-replicative, cytoplasm-targeting vector) was included for comparative purposes and HPV-16 L1 was used as a positive control (Figure 11 and 12).

Chloroplast-targeting produced the highest yields for the majority of chimaeras (Figure 9 and 11-12) and improved L1/L2 chimaera expression by up to 7-fold relative to pRIC3, and 28-fold relative to pTRAc, both which target the expressed protein to the cytoplasm (Figure 12). Statistical analysis revealed that the chloroplast-targeted L1/L2 yields were significantly higher than the cytoplasm-targeted L1/L2 yields ($p < 0.01$). However, yield differences between chloroplast-targeted chimaeras and chimaeras expressed using the self-replicative pRIC3 vector were only significant for L1/L2(56-81). These results provide evidence that pTRAkc-rbcs1-cTP is the best vector to use for the high-level production of HPV chimaeras.

2.4.2 Expression of the L1/L2 chimaeras

2.4.2.1 Highly-expressed L1/L2 chimaeras containing the HPV-16 L2 epitopes

The L1/L2(108-120), L1/L2(56-81) and L1/L2(17-36) chimaeras were highly-expressed, with yields up to 20-fold higher than the other chimaeras

(Table 4). As a result, these three L1/L2 chimaeras were chosen as vaccine antigens for the mouse immunogenicity studies.

Chloroplast-targeted L1/L2 chimaeras consistently demonstrated the highest chimaera yields of ~ 1200 mg/kg plant tissue (2-3% TSP). Although HPV-16 L1 demonstrated higher yields than the L1/L2 chimaeras, the differences were not statistically significant (Figure 12). This indicates the L2 epitopes do not significantly affect the expression and accumulation of HPV protein in chloroplasts. Furthermore, the chimaera yields are ~2-fold higher than published HPV-16 L1 yields produced using an *Agrobacterium*-mediated tobacco expression system (Maclean *et al*., 2007; Regnard *et al*., 2010) and the production of these chimaeras is commercially viable (>1% TSP; Fischer *et al*., 2004).

Assembly into higher-order structures is associated with a lower susceptibility to proteolysis (Chen *et al*., 2000) and it was hypothesized that the high accumulation of L1/L2 may be a result of assembly. The conformational-specific H16.V5 MAb binds assembled protein (Christensen *et al*., 1996) and can be used to detect assembly into higher-order structures (Carter *et al*., 2003; Wang *et al*. 2003; Ryding *et al*., 2007). All plant-expressed L1/L2 chimaeras and the HPV-16 L1 control appeared to contain a low proportion of assembled protein (<20% TSP), suggesting majority of the protein exists as unassembled L1 monomers. However, both the L1/L2(56-81) and L1/L2(17-36) chimaeras contain L2 sequences overlapping the L1 C-terminal region aa 428-483 shown to be critical for the binding of H16.V5 (Varsani *et al*., 2006b), suggesting this MAb may not be suitable for detection of chimaera assembly and cannot be used for comparable quantification. Electron microscopy will provide further insights into chimaera assembly and is analyzed in Chapter 3.

2.4.2.2 Instability of the L1/L2 chimaera with the BPV L2 aa 1-88 epitope

The L1/L2 BPV(1-88) chimaera had low expression levels in comparison to the chimaeras containing the HPV-16 L2 epitopes (Figure 6) and the E7 chimaeras (Figure 9). ELISA quantification estimated expression levels were

similar to the NSs negative control (Figure 9), although L1/L2 BPV(1-88) was detected on western blots probed with H16.J4 MAb and has achieved low yields of 40 mg/kg plant tissue (Figure 6, ELISA data not shown). In addition, the chloroplast-targeted L1/L2 BPV(1-88) was partially degraded (Figure 6D), which has been described in several HPV L1 expression studies (Hagensee *et al*., 1993; Sasagawa *et al*., 1995; Li *et al., 1997;* Kohl *et al*., 2007)

These results provide evidence that L1/L2 BPV(1-88) is not well-expressed and stable in this plant expression system. The L1/L2 BPV(1-88) chimaera contains the largest L2 sequence replacement and the 88 residue epitope replaced the entire C-terminal of L1 (Figure 2, Table 1). The HPV L1 C-terminal plays a role in VLP assembly (Zhou *et al*., 1991b; Varsani *et al*., 2006b; Bishop *et al*., 2007a), and deletion of this L1 region prevents assembly into higher-order structures which are less susceptible to degradation (Chen *et al*., 2000). Taken together, the sequence replacement of the L1 C-terminal with foreign epitope sequences is not an effective strategy for HPV chimaera expression and plants are not a viable system for L1/L2 BPV(1-88) expression.

2.4.3 Expression of the E7 chimaeras

The L1 chimaeras containing E7 epitopes had relatively low expression levels (Figure 9B) with maximum yields of 30 - 80 mg/kg plant tissue (0.3 - 0.8% TSP) were obtained (Figure 8, Table 4). Furthermore, expression was not improved using pRIC3 except for the lowest-expressing L1/L2/E7M chimaera (Figure 9B). These yields are lower than the >1% TSP threshold for the commercial production of recombinant proteins in plants (Fischer *et al*., 2004), suggesting further optimization or an alternative expression system is required for economically feasible vaccine production.

In addition, the number of epitope insertions appears to affect expression. The L1/L2/E7 chimaeras and the L1/L2(108-120) chimaera all contain the L2 aa 108-120 epitope in the h4 region of L1. However, the L1/L2/E7 chimaeras, containing an additional E7 epitope, had a up to 20-fold lower yields than

L1/L2(108-120), providing evidence that a second 8-9 residue epitope substitution into L1 negatively impacts chimaera expression (Table 4).

2.4.4 Conclusions

All the L1 chimaeras were expressed in plants using an *Agrobacterium*-mediated transient system and co-expression of the NSs silencing suppressor. Although the use of a BeYDV-based self-replicative vector (pRIC3) increases the accumulation of L1/L2 chimaeras in the cytoplasm in comparison to a non-replicative vector (pTRAc), chloroplast-targeting is the best strategy for the high-level accumulation of chimaeras in plants.

L1/L2(108-120), L1/L2(56-81) and L1/L2(17-36) were highly expressed, producing yields which were viable for commercial production (>1% TSP) and were ~2-fold higher than the maximum HPV-16 L1 yields reported in similar plant expression studies. As a result, these three L1/L2 chimaeras were selected for subsequent analysis in mouse immunogenicity studies.

Several factors should be considered in the future design of HPV chimaeras. The L1/E7 and L1/L2 chimaeras containing different epitopes inserted into similar L1 regions showed differential expression levels, providing further evidence that the expression of recombinant proteins in plants is empirical. Furthermore, comparison of the L1/L2(108-120) and L1/L2/E7 chimaeras suggests that a second 8-9 residue epitope in the E-position of L1 negatively effects chimaera expression, although this may be dependent on the size or sequence of the epitope.

Chapter 3: Purification and assembly of HPV antigens

3.1 Introduction

The L1 major capsid protein spontaneously self-assembles into virus-like particles (VLPs), which form the basis of the current prophylactic HPV vaccines (Schiller *et al*., 2008). Recombinant VLPs have been expressed in several diverse host systems including mammalian (Mossadegh *et al*., 2004), insect (Kirnbauer *et al*., 1992), yeast (Neeper *et al*., 1996), bacteria (Zhou *et al*., 1991a) and plants (Liu *et al*., 2005), with VLP sizes varying from 25-65 nm (Mach *et al*., 2006; Maclean *et al*., 2007; Kim *et al*., 2007, 2010, Park *et al*., 2008; Woo *et al*., 2008), depending on the modification of the L1 gene (Chen *et al*., 2000) and the expression host (Varsani *et al*., 2006b).

The HPV-16 L1 C-terminal helix 4 (h4) plays a role in VLP assembly and is located between residues 414-426 (Varsani *et al*., 2003a). The removal of these motifs results in capsomere formation and prevents further self-assembly into VLPs (Bishop *et al*., 2007a). Varsani *et al*. (2006b) confirmed the importance of h4, as well as the C-terminal residues 428-465. Furthermore, there are disulphide bonds between highly conserved cysteine residues 175 and 428, and mutations of these cysteines results in the formation of capsomeres rather than VLPs (Li *et al*., 1998; McCarthy *et al*., 1998; Sapp *et al*., 1998; Fligge *et al*., 2001; Varsani *et al*., 2006b). Capsomeres are thermastable, immunogenic and protect against viral challenge in animals (Rose *et al*., 1998; Yuan *et al*., 2001; Thönes *et al*., 2008; Schädlich *et al*., 2009; Jagu *et al*., 2010) and thus also have potential for development as cost-effective HPV vaccines.

Commercial HPV vaccines (currently expressed in yeast or insect cells) are expensive (Schiller *et al*., 2008), partially as a result of costly production and purification protocols (Tiwari *et al*., 2009). In addition, complicated purification methods and extensive pre-treatment can affect the stability and recovery of assembled L1 protein (Shi *et al*., 2005; Kim *et al*., 2010) and denatured L1 do not induce neutralising antibodies (Kirnbauer *et al*., 1992; Breitburd *et al*.,

1995; Suzich *et al*., 1995). As a result, the production of vaccine antigens using low-cost expression systems and simple production and purification processes remain high priorities in any commercial protein production system.

Ultracentrifugation using sucrose or caesium chloride (CsCl) density gradients and size-exclusion chromatography has been used routinely for the small-scale purification of VLPs, either for immunogenic experiments in animals or for analytical studies (Chen *et al*., 2001; Aires *et al*., 2006; Kim *et al*., 2007; Park *et al*., 2008, Woo *et al*., 2008; Schädlich *et al*., 2009). Specifically, CsCl centrifugation and sucrose sedimentation analysis are standard techniques for the quantitative separation and purification of plant-derived L1 protein (Biemelt *et al*., 2003; Maclean *et al*., 2007; Fernández-San Millán *et al*., 2008). However, these methods are unsuitable for industrial-scale production of VLPs, as they cannot be used to process large amounts of protein and require a great deal of time and labour. Furthermore, additional chromatography steps are required for commercial vaccine production, as contaminants are not completely removed by these methods (Kim *et al*., 2010).

Conventionally, several chromatography steps are required to attain highly purified protein, a strategy which is time consuming and result in the loss of the target protein (Park *et al*., 2008; Kim *et al*., 2010; Baek *et al*., 2011). Recent studies have shown the recovery, yield and purity of HPV L1 protein is improved using cation-exchange chromatography (Kim *et al*., 2010; Baek *et al*., 2011) or heparin chromatography (Kim *et al*., 2010). Purification of recombinant HPV L1 using one-step chromatography methods may reduce time, costs and labour needed for L1 purification and thus facilitate the production of HPV vaccines.

Purification of HPV L1 using heparin chromatography is based on evidence that cell-surface heparan sulphate proteoglycans (HSPG) are the primary attachment receptors for several HPV types (Joyce *et al*., 1999; Giroglou *et al*., 2001b; Combita *et al*., 2001; Drobni *et al*., 2003; Shafti-Keramat *et al*., 2003; Broutian *et al*., 2010). Heparin, structurally similar to heparin sulphate,

is a competitive inhibitor of HPV pseudoinfection (Joyce *et al*., 1999, Giroglou *et al*., 2001b; Combita *et al*., 2001; Selinka *et al*., 2002) and selectively binds assembled L1 protein (Fleury *et al*., 2009).

At least two L1 sequences interact with heparin: a C-terminal linear motif comprising of basic residues (Joyce *et al*., 1999; Bousarghin *et al*., 2003; Fleury *et al*., 2009) and a conformational binding site containing lysine residues on the assembled L1 surface (Rommel *et al*., 2005; Knappe *et al*., 2007; Fleury *et al*., 2009). HPV-16 L1 contains two basic C-terminal regions: aa 473-488 (GLKAKPKFTLGKRKAT) and aa 492-505 (SSTSTTAKRKKRKL). These regions contain a high proportion of positively-charged amino acids which comprise the nuclear localization signal (NLS) and bind DNA (Zhou *et al*., 1991b; Sun *et al*., 1995, 2010).

The importance of the C-terminal in heparin binding is of particular interest for L1 chimaeras containing C-terminal modifications. HPV-16 L1 deletion mutants that lacked the basic C-terminal sequences self-assembled into VLPs (Touzé *et al*., 2000; Fleury *et al*., 2009) and bound heparin similarly to wild-type VLPs (Fleury *et al*., 2009). Furthermore, heparin binding is lost when VLPs are denatured, confirming that heparin binds to a conformational motif on assembled L1, and that this motif is not present in the C-terminal (Giroglou *et al*., 2001b; Rommel *et al*., 2005; Wang *et al*., 2005; Fleury *et al*., 2009).

In conclusion, several methods have been used to purify HPV L1 protein, including sucrose and/or CsCl density gradients and various chromatography techniques. Multiple purification steps and extensive pre-treatment affects the stability of the protein and denatured L1 is inefficient in the production of neutralising antibodies. As a result, purification of recombinant HPV L1 protein using a simple one-step chromatography method would be ideal for the rapid and cost-effective production of HPV vaccines.

This study describes the purification of plant-expressed HPV-16 L1 and three L1/L2 chimaeras for subsequent downstream immunogenicity studies in mice.

Several size-based purification methods were examined, as well as cation-exchange chromatography and heparin chromatography.

3.2 Materials and Methods

3.2.1 Large-scale transient expression and extraction of antigens

N. benthamiana plants (2-4 weeks old) were vacuum-infiltrated with *A. tumefaciens* LBA4404 (pBIN-NSs) encoding the NSs silencing suppressor protein and the *Agrobacterium* GV3101 strain encoding HPV-16 L1 or the L1/L2 chimaeras, as described by Maclean *et al*. (2007). The plants were grown for 5 days in 16 hr light, 8 hr dark, at 22 °C.

The infiltrated leaves were harvested, weighed and ground in liquid nitrogen using a mortar and pestle. PBS extraction buffer, containing 0.5M NaCl and protease inhibitor (Roche Complete EDTA-free), was added at a ratio of 1:4 (w/v) and samples were homogenized in a Waring blender for 10 min on ice. The homogenate was sonicated on ice for 6x 20s intervals of sonication and rest (Macrotip sonication; Level 8; Heat Systems – Ultrasonics, Inc. Sonicator Cell Disruptor Model W-225 R), and the lysate was filtered through a double layer of Miracloth (CALBIOCHEM). The crude extract was clarified twice by centrifugation at 13,000 rpm for 10 min. Pellets were resuspended in 1ml PBS and stored at -70ºC. The supernatant and pellet were examined by western blotting to check for localization of the HPV antigen to the supernatant.

3.2.2 Pilot purification of HPV antigens

Several methods were examined for the purification of plant-expressed L1 vaccine antigens (Appendix B). Size-based methods such as cross-flow microfiltration and ultracentrifugation using sucrose and caesium chloride density gradients were tested, as well as single-step cation-exchange and heparin chromatography for the rapid purification of L1 and L1-based chimaeras. L1 protein extracted in PBS containing 0.5M NaCl was diluted 10x in low-salt PBS (LS PBS: 10mM NaCl PBS, pH 7.4) prior to chromatography,

to allow L1 binding to the columns. Ultrafiltration was utilized to concentrate antigens and desalt chromatography fractions for downstream application in mouse immunogenicity studies.

References for the methods used in the pilot purification experiments are given in Appendix B. Overall, purification using heparin chromatography and diafiltration using Macrosep® ultrafiltration spin tubes were the best strategies to obtain partially-purified L1 and L1-based chimaeras, and these methods were used to prepare the vaccine antigens for subsequent mouse immunological experiments.

3.2.3 Purification of vaccine antigens

3.2.3.1 Sample preparation

HPV-16 L1 and L1-based chimaeras were expressed in *N.bethamiana* and extracted as described in Section 3.2.1 using LS PBS as the extraction buffer. The double-clarified crude supernatant for L1/L2(108-120), L1/L2(56-81), L1/L2(17-36), HPV-16 L1 and the NSs-infiltrated plant extract (Vaccines 1-5 respectively) was filtered through a 0.22 µm Millipore filter prior to chromatography to remove any debris.

3.2.3.2 Heparin chromatography

Chromatography was performed using an ÄKTA Explorer System 10. The procedure was followed as recommended in the GE Healthcare column instruction manual and a flow rate of 0.5 ml/min was maintained. The column was equilibrated with 10 column volumes (cv) of low salt wash buffer (LS PBS: 10mM NaCl PBS) prior to loading the sample. The crude extract (10-20 ml) was loaded on a pre-packed 1ml HiTrap Heparin cation-exchange column (GE Healthcare, Amersham Biosciences AB, Sweden) and the column was washed with 10 cv of LS PBS wash buffer. The elution profile for each HPV antigen was optimized in a pilot experiment using a linear ionic strength gradient, whereby 10 cv of 0-100% of a high salt PBS (HS PBS) elution buffer

containing 1.5M NaCl was applied to the column. Once it had been established that all antigens eluted when <50% HS PBS was applied to the column, a 50% step elution gradient (0.75M NaCl) was applied for purification of the vaccine antigens. The step elution gradient was 10 cv of 50% HS PBS, followed by 10 cv of 100% HS PBS. The column was finally washed with 5 cv of distilled water and 5 cv of 20% ethanol. Fractions (1ml) were collected and analyzed by western dot blots.

3.2.3.3 Western dot blot detection of purified HPV antigens

The dot blots were performed as described in Chapter 2 (Section 2.2.8). CamVir1 (1:10000) was used to detect L1 and Cervarix was used as a positive control. Eluted fractions containing a high concentration of purified antigen were pooled and stored at -70ºC. For the NSs-infiltrated plant extract (V5: negative control), the fractions which corresponded with the eluted protein peak were pooled and tested on the L1 positive control vaccine (V4) dot blot, to confirm it did not contain L1.

3.2.3.4 Desalting of purified antigen samples by ultrafiltration

The purified antigens in the 50% HS PBS elution buffer (0.75M NaCl), were desalted prior to mouse vaccinations. Ultafiltration spin tubes with 10kDa MWCO filter (Macrosep® Centrifugal Devices, 10K Omega, PALL Life Sciences) were used to concentrate and desalt the purified L1 fractions rapidly by centrifuging the samples at 7000g for 15-30 min. The retentate containing the L1 antigens was diluted in LS PBS and re-concentrated by ultrafiltration several times as per the manufacturer's instructions until the samples contained a NaCl concentration similar to that found in commercial PBS (~0.15M NaCl). Both the retentate and filtrate fractions were examined

3.2.4 Analysis of antigen purity

3.2.4.1 Coomassie staining and western blot detection of HPV antigens to assess purification

The crude extract and purified sample for each of the vaccine antigens was compared by Coomassie staining and western blot analysis. Samples were prepared as described in Chapter 2 (Section 2.2.8) and equal volumes were loaded into two 10% SDS-PAGE protein gels. One gel was stained with Coomassie solution overnight at room temperature and destained 2x 2hr at 37ºC. The other gel was blotted onto nitrocellulose membrane and probed with CamVir1.

3.2.4.2 Total soluble protein quantification

The negative control vaccine (V5: NSs-infiltrated plant extract) cannot be quantified by anti-L1 western blotting. As a result, the amount of total soluble protein (TSP) was determined for each vaccine antigen using the Biorad Lowry protein assay (described in Chapter 2, Section 2.2.9) to ascertain that the TSP was similar for all vaccines.

3.2.4.3 Detection of HPV antigens by capture ELISA to determine enrichment of antigen relative to the TSP

A capture ELISA was performed as described in Chapter 2 (Section 2.2.9), using the linear epitope-specific monoclonal antibody (MAb) H16.J4. The HPV antigen yields determined by ELISA were compared to corresponding TSP yields in both the crude and purified samples to determine antigen enrichment.

3.2.5 Western blot quantification of purified vaccine antigens

A dilution series of the vaccine Cervarix (containing 40 ug/ml of insect cell-produced HPV-16 L1) was used to quantify the plant-produced HPV antigens (V1-4). Several dilutions of the antigen were analyzed to ensure quantification occurred within the linear range of the standard curve. Equal volumes of purified antigens and Cervarix dilutions were loaded into 10%

SDS-PAGE gels, proteins were blotted onto nitrocellulose membrane and the HPV antigens were detected with CamVir1 (1:10000).

Densitometry (GeneTools, Syngene, Synoptics Ltd) was used to quantify the antigens (as done by Aires *et al*., 2006; Bazan *et al*., 2009) and the amount of HPV protein present in the samples was determined using the standard curve generated by the Cervarix dilution series. Quantified HPV antigens were stored at -70ºC.

3.2.6 Structural analysis by transmission electron microscopy

Aliquots of the purified vaccine antigens were pre-treated as if they were being prepared for mouse vaccinations. The antigens were defrosted overnight at 4ºC, resuspended in PBS to the required concentration and incubated at 37ºC for 30 min.

To determine the affect of purification, the pre-treated purified antigens and the crude plant extracts for each antigen were diluted 10x in PBS, immunotrapped using CamVir1 (1:1000), a linear epitope-specific HPV-16 L1 antibody which binds both L1 monomers and assembled L1 protein (McLean *et al*., 1990), and captured on glow-discharged carbon-coated copper grids. The proteins were negatively stained with 2% uranyl acetate and viewed on a Zeiss 912 Omega Cryo EFTEM.

3.3 Results

3.3.1 Purification of plant-expressed HPV antigens

3.3.1.1 Detection of HPV antigens in the clarified extract

The localisation of L1 and the L1/L2 chimaeras to the clarified supernatant was confirmed by western blot analysis. The Coomassie-stained protein gel indicated the abundant presence of Rubisco in the supernatant and the removal of several contaminating proteins present in the pellet (Figure 1).

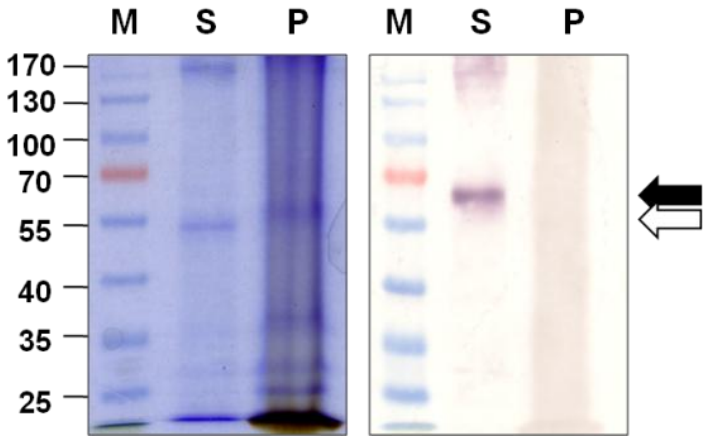

Figure 1: Localisation of the HPV antigens to the supernatant in the clarified plant extract. A) Coomassie-stained protein gel and B) Western blot detection of HPV-16 L1/L2(108-120) using the anti-L1 antibody CamVir1. Labels: M = Protein marker with size in kDa indicated on the left. S = supernatant. P = pellet resuspended in PBS. The black arrow indicates the HPV chimaera and the white arrow indicates the plant protein Rubisco.

3.3.1.2 Pilot purification of HPV antigens

Appendix B described the results from the pilot purification experiments using Coomassie-staining of proteins, as well as anti-L1 western blotting and capture ELISA using CamVir1 (data not shown). Purification using size-based techniques was largely unsuccessful and not reproducible between the vaccine antigens, as the L1/L2 chimaeras appear to assemble into a variety of structures in contrast to L1. In addition, protein degradation was detected and thus purification using chromatography was examined as an alternative method.

Although cation-exchange chromatography using the HiTrap SPFF or POROS 50HS column was unsuccessful in the purification of the L1-based chimaeras, heparin affinity chromatography purified all the vaccine antigens. The concentration and removal of salt in the chromatography fractions was examined using two ultrafiltration-based methods, either by cross-flow filtration or centrifugation spin columns. Although cross-flow ultrafiltration was effective, the method was costly with regard to time and significant protein degradation was detected, resulting in the preferential use of spin columns.

Thus, heparin chromatography and centrifugation ultrafiltration were considered the best strategies to purify the vaccine antigens for subsequent mouse immunological experiments.

3.3.1.3 Purification using heparin chromatography

Vaccine antigens were purified from the clarified crude plant supernatant by heparin chromatography using a high NaCl gradient for elution of the HPV antigens. The step elution gradient was optimised for each HPV antigen using a linear 0-100% 1.5M NaCl gradient. An example of this is shown in Figure 2A. All HPV antigens eluted between 0.45 - 0.75M NaCl (data not shown). As a result, a 50% (0.75M NaCl) step gradient was used to purify the vaccine antigens for the mouse immunogenicity study. An example of this step gradient elution with HS PBS is shown in Figure 2B. Detection of the purified HPV antigens in the eluate fractions was determined using CamVir1 dot blots.

In Figure 2, the elution of unbound protein is shown by a broad absorbance peak during the LS PBS wash. An absorbance peak was detected when the HS PBS elution buffer was applied to the column (a broad peak for the linear gradient in Figure 2A and a sharp peak for the step gradient in Figure 2B), and these fractions contained the purified HPV antigens. The chromatograms for the other vaccine antigens were similar, including the graph for the NSs-infiltrated plant extract (negative control). This indicates that the HPV antigens were co-purified with other contaminating plant proteins.

The fractions containing the partially-purified HPV antigens (or co-eluted plant proteins for the negative control) were pooled and then desalted using ultrafiltration spin columns. Western dot blots indicated that the HPV antigens were successfully retained and concentrated (data not shown).

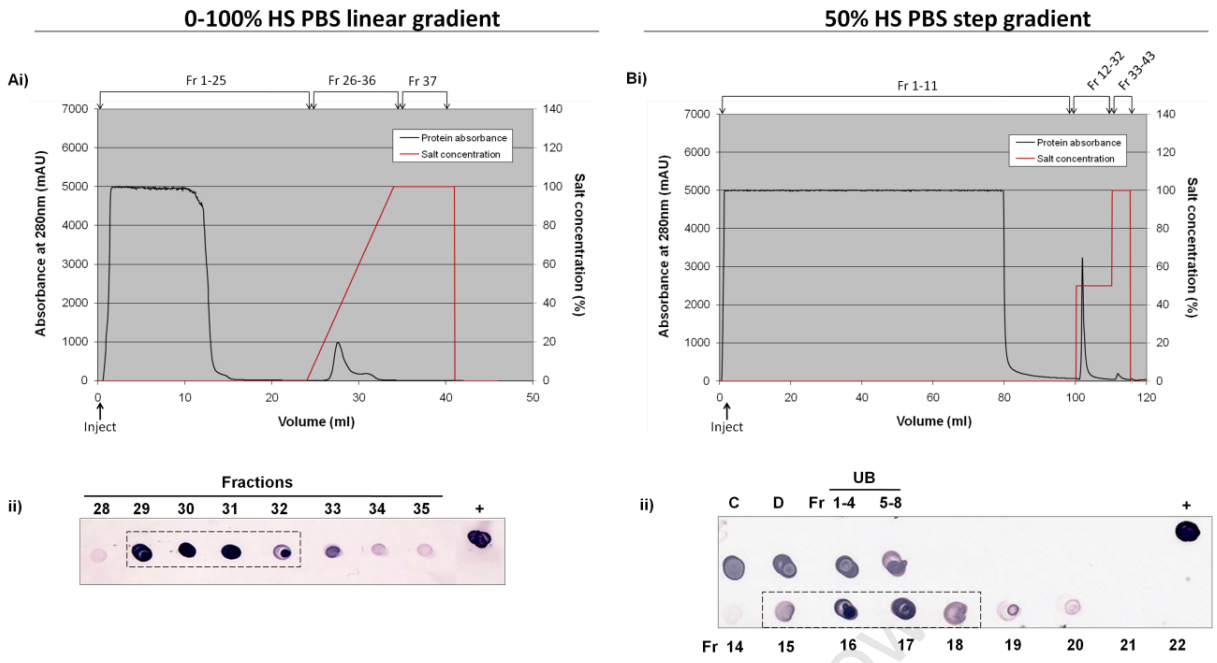

Figure 2: Purification of the plant-produced HPV antigens using heparin cation-exchange chromatography. A) L1/L2(56-81) purification using a 0-100% linear high salt elution gradient to determine the concentration of NaCl needed to elute the chimaera. B) L1/L2(108-120) purification using a 50% and 100% step high salt elution gradient. The elution of purified HPV antigens were detected in the i) chromatograms and in the ii) anti-L1 CamVir1 western dot blots. Labels: Fr = fractions. C = crude plant extract. D = crude sample diluted 10x in 1x PBS. UB = the unbound proteins eluted with the low salt wash buffer. +ve control = Cervarix. The dotted box indicates which fractions were pooled and desalted.

3.3.1.4 Purity of the vaccine antigens

The purity of the vaccine antigens was examined by comparing the purified sample to the crude plant extract. This was done using Coomassie staining, to indicate total protein present in the samples (Figure 3A), and western blot analysis, to detect the HPV antigens and indicate the loss in antigen yield (Figure 3B). Note that the L1/L2(108-120) chimaera (V1) runs higher than the other L1/L2 chimaeras (V2-3) and the L1 control (V4).

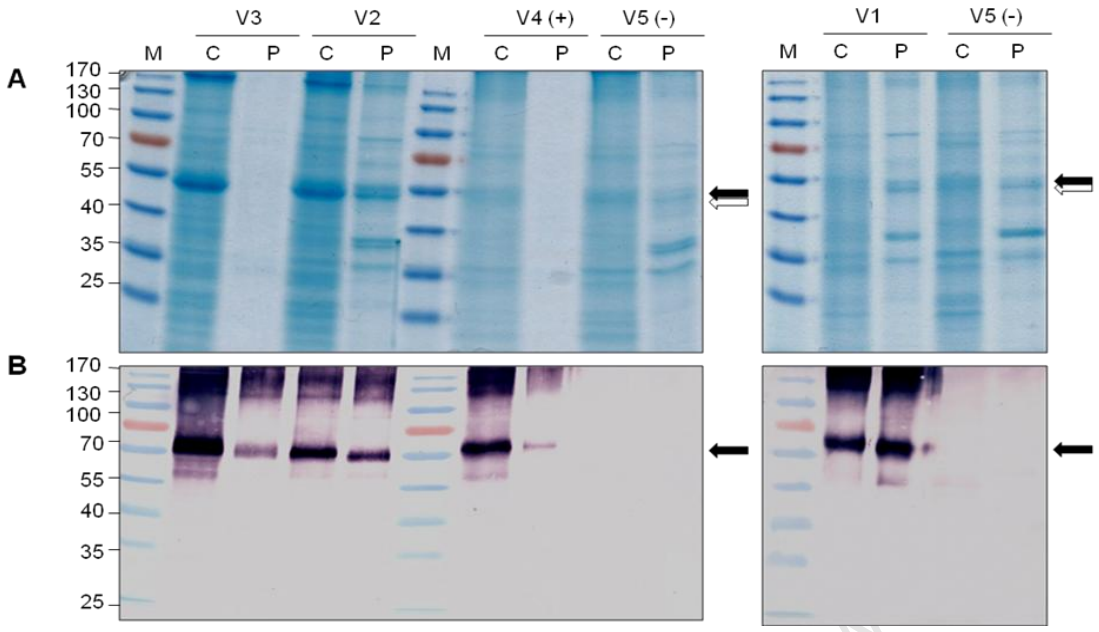

Figure 3: Purity of the plant-produced vaccine antigens. A) Coomassie-stained protein gel. B) Western blot detection of HPV antigens. M = Protein marker with size in kDa indicated on the left. C = clarified crude plant extract. P = purified antigen. V1 = L1/L2(108-120), V2 = L1/L2(56-81), V3 = L1/L2(17-36), V4 (+) = HPV-16 L1 and V5 (-) = NSs-infiltrated plant extract. The black arrows indicate the HPV antigens and the white arrows indicate the plant protein Rubisco.

Figure 3 shows the purified samples were enriched with L1 as a result of the purification procedure. The Coomassie-stained gel shows a large decrease in the total protein in the purified samples, while the western blot results indicate that there is only a small decrease in antigen yield after purification. The L1 antigen was not detected in the negative control (V5: NSs-infiltrated plant extract).

Samples were only partially-purified, as additional Coomassie-stained protein bands were detected in Figure 3A for purified antigens V1 and V3 (more concentrated than V2 and V4), thus demonstrating that the purified samples contain several contaminating plant proteins. Also, although the NSs negative control (V5) was not detected on the western blot, several similar Coomassie bands were seen in the purified NSs sample.

3.3.1.5 Enrichment of HPV antigens in purified samples

The TSP of the purified antigens was determined to: (a) ensure that the TSP was similar for the NSs negative control (containing plant proteins which were co-purified with the HPV antigens) in comparison to the other vaccine antigens, and (b) to determine HPV antigen enrichment after purification. The TSP for the purified HPV vaccine antigens (V1-4) was similar, however the TSP for the NSs plant extract negative control (V5) was almost 2-fold higher, possibly as a result of more eluate fractions being pooled or greater ultrafiltration concentration (data not shown). As a result, it was diluted accordingly in 1x PBS.

Capture ELISA, using the linear-specific H16.J4 MAb was used to estimate the amount of HPV antigen present in the crude and purified samples. To determine the effect of purification on the TSP and the enrichment of HPV antigens, the H16.J4-detected HPV yield was directly compared to the TSP yield for both the crude and purified samples (Figure 4).

Figure 4 demonstrates that purification of the plant extracts reduced both the TSP and total HPV protein, as expected. However, relative to the TSP, there is up to a 5-fold enrichment of HPV antigen in purified samples (V1-4), suggesting that heparin chromatography is effective in removing a large proportion of contaminating protein. The NSs-infiltrated plant extract (V5) showed a similar decrease in TSP with purification and the amount of TSP in the "purified" negative control lies within the levels obtained for the HPV vaccine antigens (V1-4).

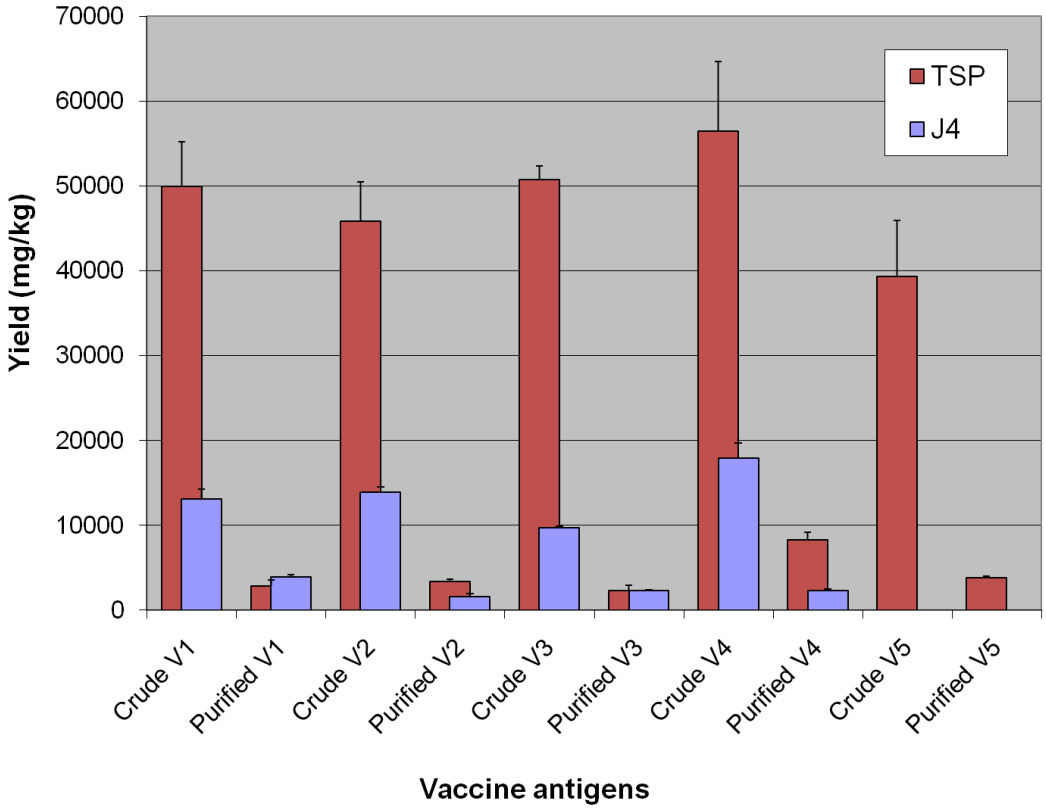

Figure 4: Total soluble protein (TSP) and total HPV protein in the crude and purified samples. TSP was determined using the Lowry assay and HPV protein was detected with H16.J4 (linear epitope-specific). V1: L1/L2(108-120), V2: L1/L2(56-81), V3: L1/L2(17-36), V4: HPV-16 L1 (positive control), V5: NSs plant extract (negative control). The error bars indicate the standard deviation.

3.3.2 Western blot quantification of purified HPV antigens

HPV antigens were quantified by western blotting using densitometry and the commercial vaccine Cervarix as the standard. An example of the western blots and standard curves used to quantify the HPV antigens are shown in Figure 5A-C.

Some L1 protein degradation, visible as a ~45 kDa band (Baek *et al*., 2011), was detected in some of the purified antigen batches, particularly after several freeze-thaw cycles. However, only the full-length 56 kDa L1 band was quantified in the samples prepared for mouse immunogenicity studies.

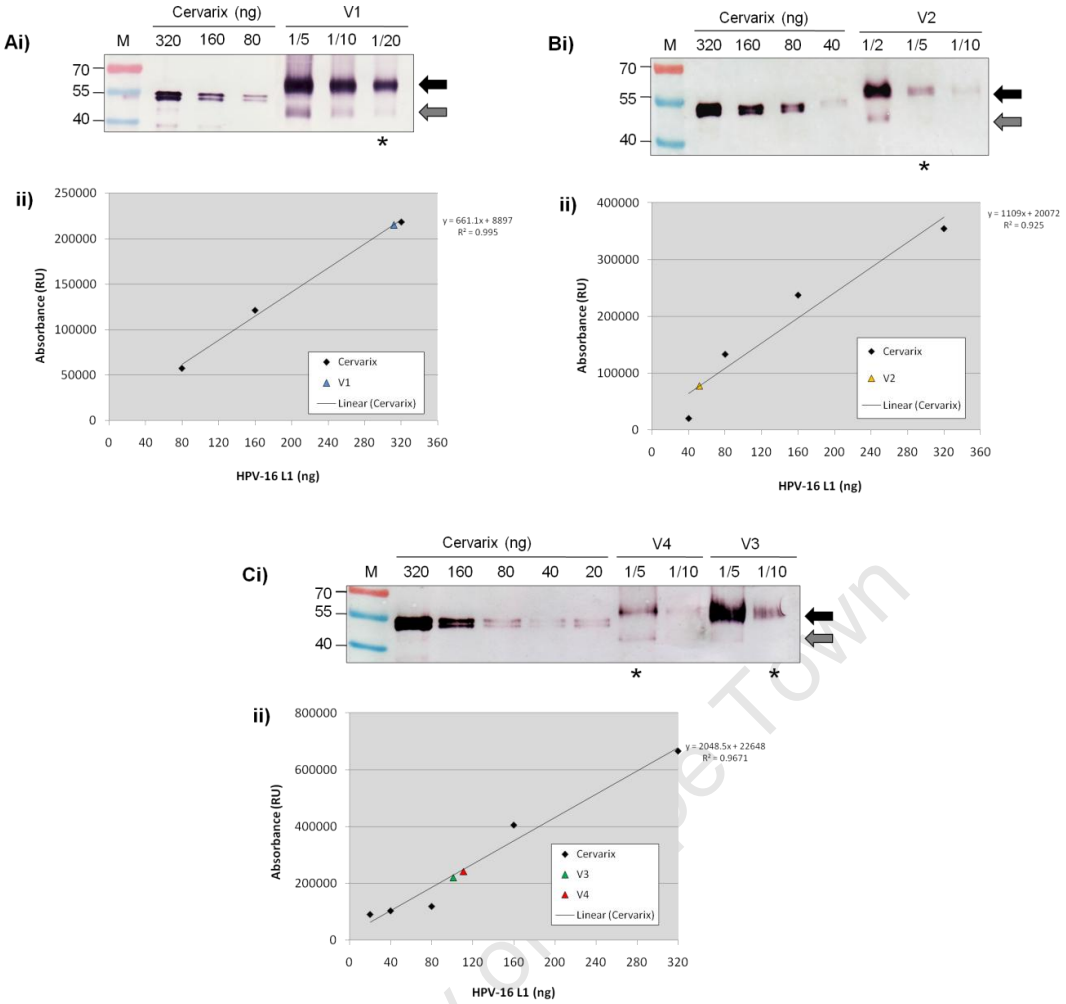

Figure 5: Western blot quantification of HPV antigens using the commercial vaccine Cervarix for A) V1: L1/L2(108-120), B) V2: L1/L2(56-81) and C) V3: L1/L2(17-36) and V4: HPV-16 L1. The i) western blots were used to generate a ii) Cervarix (HPV-16 L1) standard curve. Labels: M = Protein marker with size in kDa indicated on the left. The star indicated the antigen dilution which falls within the range of the standard curve.

3.3.3 Structural analysis of purified vaccine antigens

The structural assembly of L1 and the L1/L2 chimaeras in both the crude and purified samples was analysed by immunocapture transmission electron microscopy (Figure 6). Antigen purification resulted in the removal of contaminating background protein, particularly for L1/L2(108-120) and the negative control (Figure 6A and E respectively). In comparison to the negative control (NSs-infiltrated plant extract), all the HPV antigens appeared to contain secondary HPV structures, either capsomeres (~10 nm), capsomere aggregates, small VLPs (~25-40 nm) or full-sized VLPs (55 nm).

Purified L1/L2(108-120) assembled into small chimaeric VLPs (cVLP) which were regular in shape but varied in size (25-40 nm), while L1/L2(56-81) only appeared to contain capsomeres (Murata *et al*., 2009) and some aggregates, although VLP-like structures were visible in the crude extract. Purified L1/L2(17-36) contained a mixed population of amorphous cVLPs and a high proportion of capsomere aggregates in contrast to the crude extract, suggesting purification promoted the formation of higher-order structures. Purified V4, the HPV-L1 positive control, assembled into distinct VLPs (~50 nm), as described in previous studies (Biemelt *et al*., 2003; Maclean *et al*., 2007).

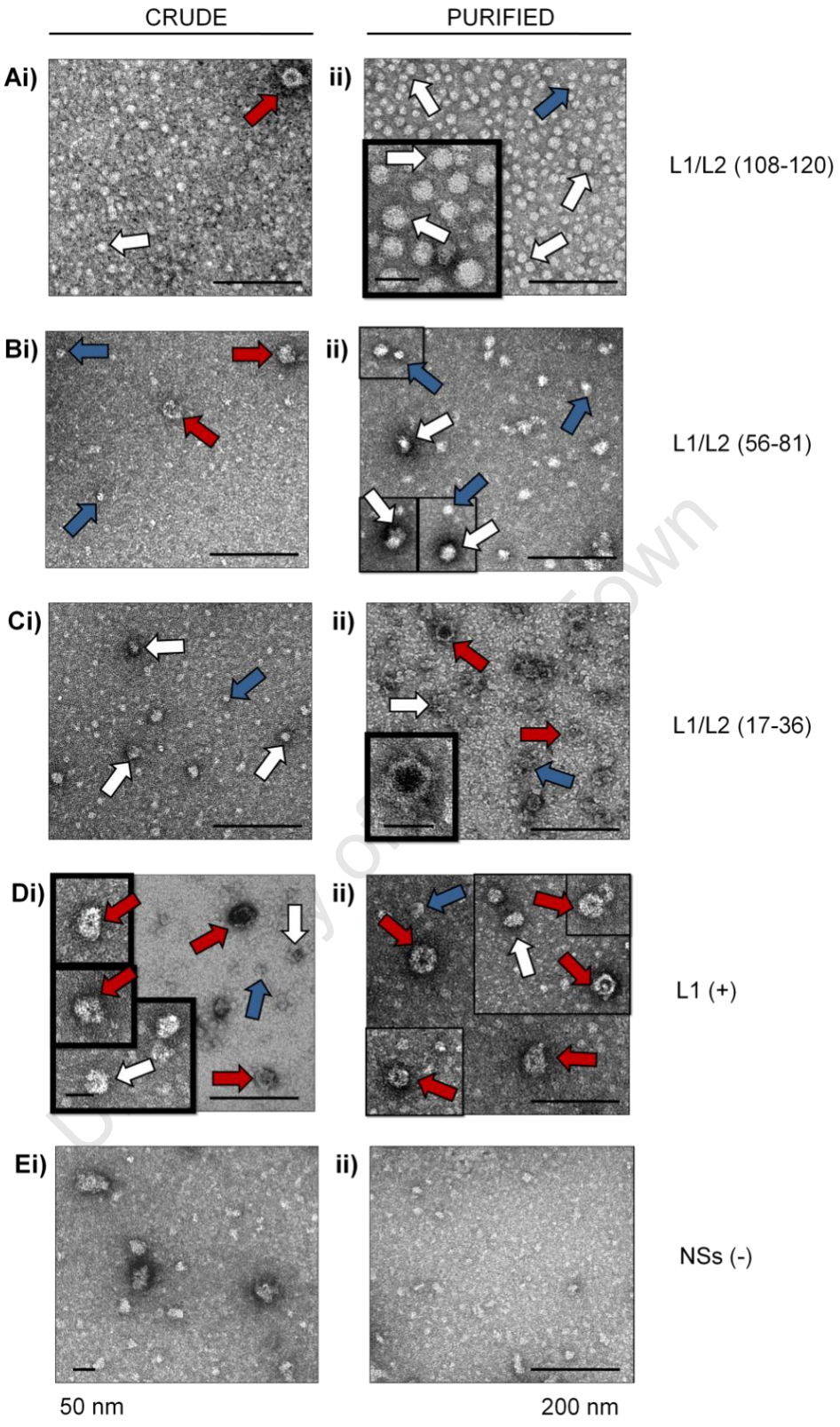

Figure 6: Transmission electron micrographs of CamVir1-immunotrapped crude and purified vaccine antigens A) V1: L1/L2(108-120), B) V2: L1/L2(56-81), C) V3: L1/L2(17-36), D) V4: HPV-16 L1 (positive control), E) V5: NSs plant extract (negative control). Grids were viewed on a Zeiss 912 Omega Cryo EFTEM. Left scale bar = 50 nm, right scale bar = 200 nm. Blue arrows indicate HPV-16 capsomeres (~10 nm), white arrows represent capsomere aggregates or small VLPs (25-40 nm) and red arrows indicate full-sized VLPs (50-55 nm).

3.4 Discussion

Stringent purification is necessary for the commercial production of vaccines, although the stability of L1 is negatively affected by multiple purification steps. Heparin affinity chromatography can be utilized to selectively purify assembled L1, and a purification strategy using a one-step chromatography method would be ideal for the rapid and cost-effective production of HPV vaccines. This study reports the purification of plant-expressed HPV-16 L1 and three L1/L2 chimaeras using heparin chromatography for subsequent immunogenicity studies in mice.

3.4.1 Optimisation of L1/L2 chimaera purification

HPV-16 L1 and the L1-based chimaeras were localized to the crude extract supernatant (Figure 1) and were purified using a variety of methods (Appendix B). Although size-dependent purification methods have been used to purify plant-expressed HPV L1 (Biemelt *et al*., 2003; Maclean *et al*., 2007; Fernández-San Millán *et al*., 2008), these methods were inefficient for L1/L2 chimaera purification and were non-reproducible between antigens. The L1-based chimaeras were broadly detected in several fractions using both sucrose and CsCl density gradient ultracentrifugation, indicating that the L1/L2 chimaeras assembled into heterologous higher-order structures, such as capsomeres, aggregates and VLPs. Furthermore, the extent of assembly appeared to differ between the chimaeras and the L1 positive control. This was confirmed by transmission electron microscopy (Figure 6), which showed distinct differences between the different L1/L2 chimaeras and the L1 control.

Chromatography was the next strategy to selectively purify HPV L1; either on the basis of surface charge, or by affinity for the proteoglycan heparin. The use of cation-exchange chromatography for the purification of yeast-expressed HPV L1 has been demonstrated using P-11 phosphocellulose (Kim *et al.*, 2009, 2010) or a POROS 50HS column (Cook *et al.*, 1999). In contrast, the plant-expressed L1/L2 chimaeras were not purified efficiently using either the strong cation-exchange HiTrap SPFF column or the POROS 50HS column (Appendix B). The majority of L1/L2 protein did not

bind to either column, although a small proportion of protein bound strongly and irreversibly to the POROS 50HS resin. This phenomenon has been described by Cook *et al. (*1999), whereby 10% of HPV-11 L1 did not bind the resin and 45-65% could not be recovered without stripping the POROS 50HS column using 0.5M NaOH.

As a result, cation-exchange chromatography was not pursued further, although the reason for the poor purification of L1/L2 chimaeras is not clear. There are two HPV-16 L1 basic C-terminal regions which contain positively charged residues: aa 473-488 and 492-505 (Zhou *et al*., 1991b; Sun *et al*., 1995, 2010). The L2 sequence insertions did not overlap the major basic regions in the C-terminal of L1 and replaced a maximum of 26 residues at aa 414-439. A possible explanation is that the overall surface charge of L1 was affected, either by the aa composition of the inserted L2 epitopes, or by differences in protein assembly. In addition, the crude plant extract may have contained several contaminating proteins which bound more strongly to the columns and out-competed HPV L1 binding.

3.4.2 Purification of the vaccine antigens

Vaccine antigens were purified using heparin chromatography (described by Joyce *et al.*, 1999; Bazan *et al.*, 2009; Johnson *et al.*, 2009; Kim *et al.*, 2009, 2010) for subsequent immunogenicity studies in mice (Figure 2). Heparin reversibly bound both the L1 and L1/L2 chimaeras in a similar manner (data not shown), and all antigens eluted with 0.75M NaCl. This is comparable to other studies where heparin-bound HPV-16 L1 eluted between 0.5 - 1.2M NaCl (Bazan *et al*., 2009; Kim *et al*., 2010; Baek *et al*., 2011).

Heparin selectively purifies assembled L1 protein by binding to a conformational motif which is not present on the C-terminal of L1 (Fleury *et al*., 2009) and is unaffected by the L2 sequence replacements. This is particularly beneficial for vaccine production, as denatured L1 does not elicit the production of neutralising antibodies (Kirnbauer *et al*., 1992; Suzich *et al*., 1995; Breitburd *et al*., 1995). Furthermore, Kim *et al.* (2010)

demonstrated that purification of HPV-16 L1 using heparin chromatography gave high recovery yields (~60%) and produced immunogenic VLPs (25-65 nm in diameter).

The purity of the heparin-purified samples was examined by Coomassie staining and western blot detection of L1 using CamVir1 (Figure 3). The purified samples were enriched with L1 or L1/L2 chimaeras, as there was a significant decrease in total protein with a relatively small decrease in antigen yield when compared to the crude samples. This was confirmed by H16.J4 capture ELISA and TSP assays (Figure 4).

Samples were partially-purified and contained several contaminating plant proteins (V1 and V2, Figure 3A), also present in the purified negative control (V5, Figure 3A). Contaminants were also observed in the purification of yeast-expressed HPV-16 L1 using heparin chromatography (Kim *et al.*, 2010). As a result, a single step method using heparin chromatography is not sufficient to obtain highly-purified HPV L1 and L1/L2 chimaeras. Kim *et al.* (2010) demonstrated that co-purified contaminating proteins from yeast were not completely removed by additional cation-exchange and hydrophobic interaction chromatography steps, suggesting many of the contaminants have similar isoelectric points and hydrophobicity profiles to L1. Furthermore, the additional chromatographic steps reduced L1 recovery to ~10%. However, pure HPV-16 L1 was obtained by ammonium sulphate precipitation of yeast-expressed HPV-16 L1 prior to heparin chromatography, a method which should be examined in further purification studies using plant-expressed HPV L1-based proteins.

3.4.3 Western blot quantification of antigens

The purified antigens were quantified by western blot analysis (discussed by Heidebrecht *et al*., 2009) using densitometry to measure the intensity of the CamVir1-detected L1 bands and the commercial vaccine Cervarix as the HPV-16 L1 standard (Figure 5).

L1 degradation was detected in some of the batches of purified antigen, particularly after several freeze-thaw cycles. This was seen at high concentrations of V1, V2 and V4 in Figure 5. However, the majority of the antigen proteins were not degraded and only the full-sized 56 kDa L1 band was quantified to ensure mice were immunized with similar doses of full-length antigen. Other groups have reported similar HPV-16 L1 degradation patterns when expressed in insect cells (Kirnbauer *et al*., 1992), yeast (Cook *et al*., 1999) and bacteria (Zhang *et al*., 1998). A consideration for future purification studies is the salt concentration of the extraction and diafiltration buffers, as VLP disassembly occurs in low-salt conditions (Murata *et al*., 2009). Increasing the salt concentration to 0.5 or 1M NaCl may stabilize VLPs (Mach *et al*., 2006) and reduce degradation observed in the purified samples (Figure 5).

3.4.4 Assembly of the vaccine antigens

The assembly of plant-derived HPV-16 L1 and the L1/L2 chimaeras was analysed using immunocapture electron microscopy (Figure 6). Purification appeared to remove some background protein and all the plant-expressed L1/L2 chimaeras and the L1 positive control assembled into higher-order structures such as capsomeres, aggregates and VLPs.

Plant-expressed HPV-16 L1 VLPs are typically 30-65 nm in size (Biemelt *et al*., 2003; Varsani *et al*., 2003b; Liu *et al*., 2005; Varsani *et al*., 2006a; Maclean *et al*., 2007), with the majority of VLPs reported as 50-60 nm in diameter when localised to the tobacco chloroplasts (Maclean *et al*., 2007; Fernández-San Millán *et al*., 2008; Lenzi *et al*., 2008). In this study, HPV-16 L1 assembled into full-sized VLPs (~50 nm, Figure 6Dii).

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Assembly of chimaeras into VLPs appears to be affected by the length of the L2 epitope, with L1/L2(108-120), L1/L2(17-36) and L1/L2(56-81) containing 13, 20 and 26 residue epitope replacements respectively. Plant-expressed L1/L2(108-120), with the shortest L2 epitope, successfully assembled into distinct cVLPs, although they were generally smaller than L1 VLPs (Chen *et*

al., 2000) and varied greatly in size (25-40 nm, Figure 6A). In contrast, L1/L2(17-36) predominantly formed capsomere aggregates, although the presence of larger amorphous VLP-like structures suggest there may be partial-assembly of VLPs (Figure 6C). Finally, L1/L2(56-81) with the longest L2 epitope predominantly assembled into capsomeres, with no VLP-like structures observed (Figure 6B).

L1/L2(108-120) has also been expressed in insect cells and the CsCl-purified chimaera was shown to assemble into amorphous VLPs and capsomere aggregates (Varsani *et al*., 2003a). It is not clear why HPV VLPs produced in various hosts are of different diameters, although it is postulated that differences between prokaryotic and eukaryotic systems may be a result of post-translational modifications (Varsani *et al*., 2006b). However, the VLPs formed by the plant-expressed L1/L2(108-120) chimaera resemble insect cell-expressed HPV-33 L1 VLPs purified using a two-step chromatography strategy (Baek *et al*., 2011), suggesting the morphology may be a result of chromatography purification and not the expression system.

Capsomere assembly was expected as the deletion of L1 residues 428-465 or a mutation of the cysteine residue at aa 428 eliminated assembly of capsomeres into VLPs (Varsani *et al*., 2006b). Both the L1/L2(17-36) and L1/L2(56-81) chimaeras contain L2 sequence replacements which overlap this region, with epitopes replacing aa 414-433 and 414-439 respectively. Furthermore, the L2 epitopes replaced the h4 region which is involved in VLP assembly (Varsani *et al*., 2003a). However, capsomeres are immunogenic (Rose *et al*., 1998; Yuan *et al*., 2001; Fligge *et al*., 2001; Schädlich *et al*., 2009) and provide an alternative thermastable antigen for second-generation HPV vaccines.

3.4.5 Conclusions

Plant-expressed HPV-16 L1 and L1/L2 chimaeras were purified using heparin chromatography and quantified by anti-L1 western blot analysis. The vaccine antigens assembled into a variety of heterologous higher-ordered structures

such as capsomeres, aggregates and VLPs, which rendered size-based purification techniques non-reproducible between antigens. Plant-expressed HPV-16 L1 assembled into full-sized VLPs which were similar in size and morphology to those expressed in other systems. The L1/L2(108-120) chimaera assembled into small cVLPs, which were regular in shape but varied in size, while the L1/L2(17-36) chimaera predominantly assembled into capsomere aggregates. L1/L2(56-81) did not assemble into cVLPs and appeared to exist exclusively as capsomeres. These results provide further evidence that the C-terminal h4 region plays a role in VLP assembly and that sequence replacements >13 residues negatively affects VLP assembly. Assembled L1 protein, existing either as VLPs or capsomeres, has been shown to elicit neutralising antibodies and the immunogenicity of the vaccine antigens was further examined in mice.

Chapter 4: Immunogenicity of L1/L2 chimaeras

4.1 Introduction

Vaccines antigens are selected on the basis of their immunogenicity. The humoral immune response, which involves the production of neutralising antibodies (NAb) against foreign antigens, is the most important factor in prevention of PV infection (Breitburd *et al*., 1995; Suzich *et al*., 1995; Kirnbauer *et al*., 1996). Immunisation with VLPs elicits Nabs, which are predominantly directed against immunodominant L1 epitopes (Kirnbauer *et al*., 1992; Roden *et al*., 1994; Roden *et al*., 2000), although low-titre antibodies against L2 have been shown to neutralise pseudovirions *in vitro* (Roden *et al*., 2000, Kawana *et al*., 2003).

VLPs are comprised of assembled L1 capsomeres (Buck *et al*., 2008; Chen *et al*., 2000), and L1 modifications may influence assembly into higher-order structures. This is of particular interest in the development of L1-based chimaera vaccines, as L1 immunogenicity is dependent on L1 assembly into capsomeres and VLPs (Thönes *et al*., 2008; Schädlich *et al*., 2009) and denatured L1 does not elicit detectable immune responses (Fernández-San Millán *et al*., 2008; Schädlich *et al*., 2009)

L1/L2 chimaeras containing L2 epitopes in the h4 region of L1 has been shown to affect VLP assembly (Varsani *et al*., 2003a; Bischop *et al*., 2007a; Schädlich *et al*., 2009; Murata *et al*., 2009). However, capsomeres elicit the production of NAbs and protect against challenge in animal models (Rose *et al*., 1998; Yuan *et al*., 2001; Ohlschläger *et al*., 2003; Dell *et al*., 2006; Thönes *et al*., 2008), and the low immunity of capsomeres can be improved by use of an adjuvant (Thönes *et al*., 2008; Schädlich *et al*., 2009; Jagu *et al*., 2010). In addition, assembly of L1 into “small” T=1 VLPs (30-40 nm) enhances humoral immunity and elicits antibody levels comparible to full-sized T=7 VLPs (Schädlich *et al*., 2009).

The host-specificity of papillomaviruses (PV) has complicated the development of HPV candidate vaccines, as the demonstration of protective efficacy in animal systems is a pre-requisite for human clinical trials (Rybicki, 2009). Animal PV vaccines can be used in live animal challenge studies to directly test the protective ability of a vaccine provide a proof of concept.

Two studies using cotton tail rabbit papillomavirus (CRPV) have demonstrated proof of efficacy for plant-produced PV L1 vaccines in a rabbit model. Immunisation with partially-purified CRPV L1 capsomeres (Kohl *et al*., 2006) and chimaeric tobacco mosiac virus (TMV) particles carrying a CRPV L2 peptide (Palmer *et al*., 2006) successfully protected rabbits against CRPV challenge and prevented papilloma formation. Furthermore, the efficacy of plant-derived CRPV L1 compared favourably with insect cell-produced CRPV L1 VLPs used as a positive-control (Kohl *et al*., 2006) and in a parallel study (Govan *et al*., 2006).

Antisera from immunized animals can be assayed to detect antibody responses against HPV L1 and L2 and thus give an indication of vaccine immunogenicity. Analysis of several plant-derived HPV immunogenicity studies revealed that low doses of plant-derived HPV-16 L1 elicited weak immune responses when administered subcutaneously (0.04 – 4 µg; Biemelt *et al*., 2003; Varsani *et al*., 2003b; Varsani *et al*., 2006a). However, 1-3 doses of 5-11 µg L1 co-administered with Freund's adjuvant is sufficient to elicit detectable immune responses in mice (Maclean *et al*., 2007; Fernández-San Millán *et al*., 2008; Paz De la Rosa *et al*., 2009).

Although a challenge model for HPV does not exist, the antibody-mediated neutralisation of HPV pseudovirions (PsVs) *in vitro* can be used to detect HPV-specific antibody responses in antisera, and give an indication of vaccine immunogenicity. PsVs can be produced in mammalian cells and consist of the co-assembled L1 and L2 capsid proteins encapsidating a reporter plasmid (Pastrana *et al*., 2004). Human embryonic kidney cells over-expressing the SV40 large T antigen (HEK293TT) are co-transfected with a plasmid encoding the codon-modified HPV L1 and L2 capsid genes, and a reporter gene

plasmid, both containing a SV40 origin of replication. Expression of L1 and L2 results in the assembly of PsVs and the encapsidation of the small reporter gene plasmid (Buck *et al*., 2004). The PsVs can be harvested from the cells and purified for use in a neutralisation assay.

PsV infection of the HEK293TT cells occurs in a similar manner as infection with native HPV virions and ultimately results in the expression of the reporter gene. The reporter gene used in this study was secreted alkaline phosphatase (SEAP) and the enzyme can be detected in the extracellular medium by the addition of a substrate (Buck *et al*., 2004). In the assay, PsVs are pre-incubated with sera and antibodies against HPV neutralise the PsVs and thus prevent subsequent cell infection and SEAP expression. As a result, the antibody-mediated neutralisation of PsVs is indirectly detected as a reduction in SEAP activity using a highly sensitive chemiluminescent reporter system (Pastrana *et al*., 2004).

Previous studies have shown that plant-derived HPV-16 L1 (Maclean *et al*., 2007; Fernández-San Millán *et al*., 2008) and L1-based chimaeras (Paz De la Rosa *et al*., 2009) assembled into immunogenic VLPs and elicited the production of NAb, the gold standard for demonstrating the potential of prophylactic HPV candidate vaccines (Rybicki, 2010). Furthermore, insect cell-produced L1/L2 chimaeras were immunogenic and elicited both anti-L1 and anti-L2 humoral responses (Varsani *et al*., 2003a). As a result, plant-derived L1/L2 chimaeras with L2 epitopes inserted in the same h4 region of L1 may also be immunogenic and elicit the production of both NAb against homologous HPV-16 and cross-neutralising antibodies against other HPV types.

In this study, mice were immunized with plant-derived L1 and three L1/L2 chimaera candidate vaccines containing the cross-neutralising L2 epitopes aa 108-120, 56-81 and 17-36. The immunogenicity of the chimaeras was analysed with respect to chimaera assembly and their ability to elicit anti-L1, anti-L2 and protective NAb against homologous HPV-16 and heterologous HPV-18, 45 and 52 PsVs was investigated.

4.2 Materials and Methods

4.2.1 Immunisation of mice

Female C57/BL6 mice from the South African Vaccine Producers Animal Unit (Johannesburg, South Africa) were maintained under Biosafty Level 2 (BSL-2) conditions in the Animal Unit in the Health Science Faculty, University of Cape Town. Permission for this study was granted by the Research Ethics Committee, University of Cape Town (AEC 008/037).

Mice (7-8 weeks old) were immunised to test humoral antibody responses to plant-derived HPV-16 L1/L2 candidate vaccines. The controls included plant-expressed HPV-16 L1 (positive control) and NSs-infiltrated plant extract (negative control). The L1/L2(108-120) chimaera (published as SAF; Varsani *et al.*, 2003a) has been shown by our laboratory to illicit anti-L1 responses and thus served as an additional positive control. The vaccination details are shown in Table 1.

Table 1: Plant-derived vaccine antigens used in the immunogenicity study

Vaccine	Vaccine No. (*n=10)	Group No. (*n=5)	Antigen dose (µg)	†TSP (mg/ml)
L1/L2(108-120)	V1	G1 & G2	10	0.12
L1/L2(56-81)	V2	G3 & G4	10	0.14
L1/L2(17-36)	V3	G5 & G6	10	0.09
HPV-16 L1 (+)	V4	G7 & G8	10	0.33
Plant extract (-)	V5	G9 & G10	N/A	0.16

*n = number of mice
†TSP = total soluble protein

The purified vaccine antigens were adjusted to contain a 10 µg dose in 100 µl Dulbecco's PBS (DPBS; Sigma). The total soluble protein (TSP) in each vaccine was assessed using a Bradford protein assay (Chapter 2, Section 2.2.9) to ensure the negative vaccine control contained a similar TSP in comparison to the other HPV vaccines (Table 1). The vaccine was prepared by homogenization of the vaccine antigen in Freund's Incomplete Adjuvant (FIA) in a 1:1 volume ratio using the syringe-extrusion technique (Koh *et al.*, 2006).

Mice were divided up into 2 groups of 5 mice per vaccine and were subcutaneously injected into the right flank, left flank or the inguinal site. Pre-bleeds were taken 12 days prior to vaccination (Day 0) and mice were boosted on Day 13, 27, 41 and 48 (approximately every 2 weeks, except for Day 48 when it was decided to boost rather than obtain a test bleed) before obtaining the final bleeds at Day 62 (~9 weeks post-vaccination). Serum was isolated and stored at -70°C.

4.2.2 ELISA detection of anti-L1 antibodies in mouse sera

4.2.2.1 Preparation of the insect cell-produced HPV-16 L1

Insect cell-produced HPV-16 L1 (provided by Gillian de Villers) was used as an ELISA antigen to detect anti-L1 antibodies in the mouse sera. Insect cell-expressed L1 was used instead of plant-expressed L1 to avoid the background detection of antibodies against contaminating plant proteins. *Spodoptera frugiperda* (*Sf-9*) cells were grown shaking in SF90011 serum-free medium (Gibco) at 27ºC and infected at a multiplicity of infection (MOI) of 1.0 and a cell density of $1x10^6$ cells/ml. Cells were harvested after 96 hrs by centrifugation (1000 x g for 5 min) and pellets were washed with DPBS and stored at -70ºC.

HPV-16 L1 was extracted by resuspending cells to 4×10^6 cells/ml in high-salt PBS (0.8M NaCl 1x PBS) containing protease inhibitor (Roche Complete EDTA-free) and sonicating on ice for 5x 20s intervals of sonication and rest (Microtip sonication; Level 5; Heat Systems – Ultrasonics, Inc. Sonicator Cell Disruptor Model W-225 R). The cell lysate was clarified by centrifugation (5000g for 5 min) to remove cell debris and the centrifugation step was repeated using the supernatant. The commercial vaccine Cervarix (20 µg HPV-16 L1) was used as a HPV-16 L1 standard for western blot quantification of HPV-16 L1 (as described in Chapter 3, Section 3.2.5) and L1 was detected with CamVir1 (1:10000; Abcam®).

4.2.2.2 ELISA detection of anti-L1 antibodies

The anti-L1 antibody titre was determined by direct ELISA. A 96-well Maxisorp microtitre plate (Nunc) was coated with 100µl/well (30 ng) of insect cell-produced HPV-16 L1 antigen diluted in 1x PBS and incubated overnight at 4°C. Plates were blocked with blocking buffer (1% skim milk in 1x PBS; 200ul / well) for 2 hrs at room temperature and then washed 4x with PBS.

Mouse sera were pooled into vaccines (10 mice / vaccine) for analysis. Final bleed mouse sera were diluted in blocking buffer in a 4-fold series in triplicate, ranging from a dilution of 1:50 to 1:51200. Pooled pre-bleed sera were tested at 1:50 dilution and served as a negative control. Diluted sera was added to the wells (100µl / well) and incubated for 2 hrs at room temperature. Positive controls wells contained 1:50 dilution of anti-L1 antibodies; both CamVir1 (Abcam®), which binds both linear and conformational epitopes (McLean *et al*., 1990), and H16.V5 MAb, which binds specifically to conformational epitopes (Christensen *et al*., 1996). Blank wells with no antibody were included as a background control.

After a 4x PBS washing step, goat anti-mouse horseradish peroxidase conjugate (1:2000; Sigma) diluted in blocking buffer was added to the wells (100 ul / well) and incubated for 1 hr at 37ºC. Plates were washed 4x with PBS (200µl / well) and 100 ul of O-phenylenediamine dihydrochloride (OPD) (DAKO; Denmark) was added per well. Plates were developed in the dark for 30 min at room temperature, the reaction was stopped with 0.5M H_2SO_4 and the absorbance at 490nm was detected. The anti-L1 binding titres were expressed as a reciprocal of the maximum serum dilution which produces higher absorbance readings than that of the corresponding pre-bleed serum diluted at 1:50.

4.2.2.3 Statistical analysis

A two-tailed, non-paired t-test was used to calculate statistical significance of the final bleed anti-L1 response, as compared to the negative control vaccine (p = 0.01). One-way Analysis of Varience (ANOVA) was used to compare the

vaccines and the Fisher LSD, Turkey HSD and Bonferroni tests were used to determine the significance (p = 0.01).

4.2.3 Western blot detection of anti-L2 antibodies

4.2.3.1 Preparation of E. coli-produced HPV-16 L2

His-tagged HPV-16 L2 protein produced using the pProEX htb vector in *E. coli* (provided by David Mutepfa) was used for the western blot detection of anti-L2 antibodies in mouse sera. *E. coli* cultures were grown shaking at 37ºC to an OD_{600} of 0.6 and then induced by the addition of 0.6 mM iso-propyl-β-thiogalactoside (IPTG). After 3 hrs, cells were harvested by centrifugation (3800g for 15 min at 4ºC) and the pellet was retained and weighed.

The inclusion bodies were extracted by resuspension of the cells in 4 volumes of lysis buffer (50 mM Tris pH 8.5, 5 mM β-mercaptoethanol) and phenylmethanesulfonyl fluoride (PMSF) and lysozyme (Roche) was added to a final concentration of 0.4 mM and 0.08 µg/µl respectively. The cells were incubated on ice for 20 min, Triton-X was added to 1% and cells were further incubated for 20 min at 37ºC until the solution was viscous. DNase and RNase were added to 4 µg/ml and 40 µg/ml respectively and cells were incubated for 30 min at room temperature until viscosity cleared.

The inclusion bodies were collected by centrifugation at 13,000 rpm in a microcentrifuge for 15 min at 4ºC and the pellet resuspended in 1ml lysis buffer (2.5 mM Tris pH 8.0, 3.125 mM β-mercaptoethanol, 0.2 mM EDTA, 0.0025% Triton-X) and left to lyse for 10 min at room temperature. The sample was centrifuged at 13,000 rpm for 15 min at 4ºC and pellets were washed 4x with PBS. The pellet was resuspended in 1 volume PBS of the weight of pellet, quantified by Coomassie staining using a bovine serum albumin (BSA) standard and stored at -20ºC.

4.2.3.2 Western blot detection of anti-L2 antibodies

The *E. coli*-produced HPV-16 L2 antigen was incubated at 95°C for 5 min in 5x loading buffer and was loaded into a 10% SDS-PAGE gel. Instead of using a 10-well comb, a 2-well comb was used: a small well for the protein marker and a large well consisting of the 9 wells fused together, thus producing a single wide well which allowed the protein to spread equally across the width (Figure 1).

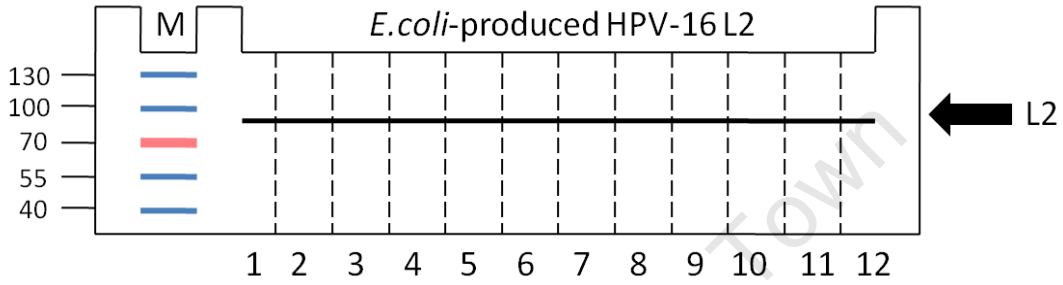

Figure 1: Schematic diagram of the western blot technique used to detect anti-L2 (~80 kDa) antibodies in mouse sera. M = protein marker with the protein sizes indicated on the left. Strip 1-12 = membrane strips used to individually probe mouse sera.

E. coli-expressed His-tagged HPV-16 L2 antigen (2.5 mg) was separated on a 10% SDS-PAGE gel (Sambrook *et al.*, 1989) and transferred onto a nitrocellulose membrane by semi-dry electroblotting as described in Chapter 2, Section 2.2.8. The western blotting protocol was then modified, whereby the portion of the membrane between 55-130 kDa containing the L2 protein (~80 kDa) was divided into 12 similar-sized strips to probe with different sera. The membrane strips were transferred into individual wells in a 25-well tissue culture plate and incubated in blocking buffer for 4 hrs at room temperature.

Individual pre-bleed and final bleed mouse sera were pooled into vaccines (10 mice per vaccine) and the membrane strips were probed with positive control mouse anti-His antibody (1:2000, Serotech) or pooled mouse sera diluted 1:100 in blocking buffer. Sera were added to different wells and incubated shaking overnight at room temperature. The strips were then washed 4x 10 min with blocking buffer and then probed with secondary goat anti-mouse IgG antibody conjugated to alkaline phosphatase (1:5000; Sigma) for 2 hrs at

room temperature. The individual strips were washed again for 4x 10 min with wash buffer and then developed with NBT/BCIP (Roche).

Densitometry (GeneTools, Syngene, Synoptics, Ltd) was used to measure the absorbance intensity of each L2 band. Values were normalized for non-specific background absorbance using the value associated with the negative control vaccine. Sera with L2 bands having absorbance values >2x the value observed in the HPV-16 L1 final bleeds elicited an anti-L2 response.

4.2.4 HPV pseudovirion neutralisation assays

4.2.4.1 Preparation for the neutralisation assays

The protocols used for the HPV pseudovirion (PsV) neutralisation assays are taken from Dr John Schiller's Lab of Cellular Oncology technical files (Website 3: http://home.ccr.cancer.gov/Lco/) and the HPV L1/L2 pSheLL plasmids and the pYSEAP reporter plasmid were kindly provided by Dr John Schiller.

The pYSEAP plasmid was checked using a *Sal*I and *Bam*HI restriction enzyme digest (as described in Chapter 2, Section 2.2.4.3). The HPV L1/L2 pSheLL plasmids were similar in size and have similar restriction enzyme sites, thus the plasmids were sequenced to confirm their identity using two sets of pSheLL vector-specific primers which bind upstream and downstream of the HPV L1 and L2 genes (Table 2). Sequences were aligned with the HPV L1/L2 pSheLL plasmid sequence and HPV L1 or L2 gene sequences using DNAMAN sequence analysis software.

Table 2: pSheLL vector-specific sequencing primers

Sequencing target	Primer	Sequence	T_m (°C)	Size (nt)
HPV L1	L1 Fwd	TGACCTTATGGGACTTTCCTAC	56.3	22
	L1 Rvs	CACCATAAGCAGCCACAAT	55.5	19
HPV L2	L2 Fwd	TACCACCACGAACAAGCAC	57.5	19
	L2 Rvs	AAGCCATACGGGAAGCAA	55.4	18

Plasmids maps of pYSEAP and the HPV L1/L2 pSheLL plasmids used in PsV production is shown in Figure 2. The restriction enzymes sites and the location of the sequencing primers (L1 and L2 Fwd and Rvs) are indicated on the pYSEAP and pSheLL plasmids.

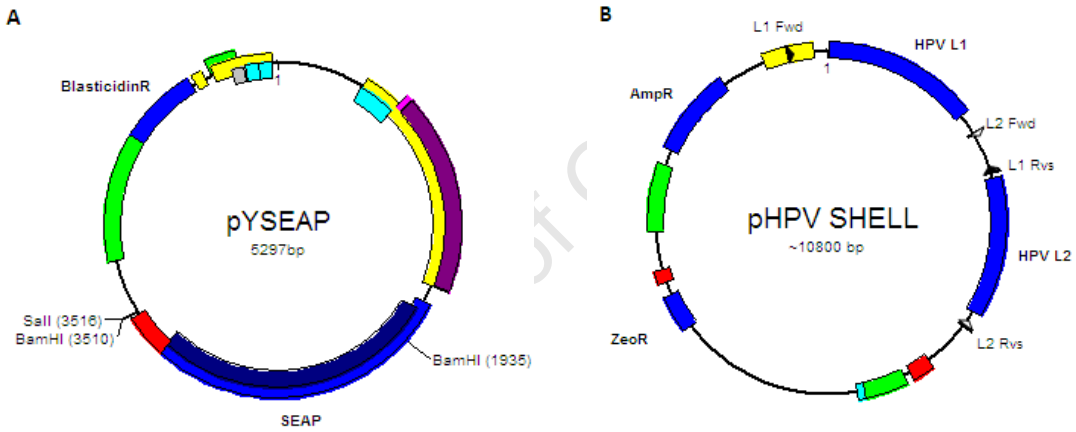

Figure 2: Schematic maps of the plasmids used in the HPV pseudovirion assays. A) pYSEAP reporter plasmid, showing the *Bam*HI and *Sal*I sites used in the restriction digest analysis. B) Generalized map of the HPV L1/L2 pSheLL plasmid vectors. The p16, p18, p45 and p52 SheLL plasmids are similar in structure, containing both L1 and L2 genes as well as the antibiotic markers for Zeo and Amp resistance. They differ in that they contain the HPV type-specific L1 and L2 genes, and p16 and p45 SheLL contain an enhancer element not present in p18 and p52 SheLL. The primers used to sequence the L1 and L2 genes are shown. The map colours represent the following: blue = CDS region, dark blue = mature peptide, green = origin of replication, red = poly A signal, turquoise = enhancer element, yellow = promoter, grey = repeat region, purple = intron and pink = exon.

Endotoxin-free plasmid DNA (NucleoBond® Xtra Midi EF, Macherey-Nagel) was prepared from *E. coli* cultures grown under the appropriate antibiotic selection for both the pYSEAP plasmid and HPV-16, 18, 45 and 52 pSheLL plasmids (Table 3) and DNA was stored at -70ºC.

Table 3: HPV PsV neutralisation assay plasmid vectors used in this study

Plasmid	HPV type	Gene of interest	Size (bp)	Antibiotic resistance
p16 SheLL	HPV-16	L1 & L2	10827	Ampicillin (100 µg/ml)
p18 SheLL	HPV-18	L1 & L2	10723	Ampicillin (100 µg/ml)
p45 SheLL	HPV-45	L1 & L2	10814	Ampicillin (100 µg/ml)
p52 SheLL	HPV-52	L1 & L2	10725	Ampicillin (100 µg/ml)
pYSEAP	-	SEAP	5297	Blasticidin (75 µg/ml)

4.2.4.2 Transfection of HEK293TT cells

The HEK293TT cell line was kindly provided by Dr John Schiller. HPV PsVs were produced as described in the "Production of Papillomaviral Vectors (Pseudoviruses)" protocol revised in June 2010.

HEK293TT cells were cultured in complete high glucose Dulbecco's Modified Eagle Medium (cDMEM) containing 1% GlutaMAX™ (Gibco) and 10% fetal calf serum (Gibco). The cDMEM media was supplemented with 1% non-essential amino acids (Gibco), 100 units/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco), 10 µg/ml Fungin™ (InvivoGen) and 250 µg/ml Hygromycin B (Roche) to select for the TT antigen (cDMEM-Ab). The thawing and passaging of cells was done as described in the protocol.

Cells were pre-plated in cDMEM (without antibiotics or Hygromycin B) in a 175cm^2 flask to reach 50-70% confluence the following day. On the day of transfection, fresh cDMEM was added to the cells and aliquots of endotoxin-free plasmid DNA were thawed on ice. The transfection mix was prepared as follows: 175 ul FuGene6 (Roche) was added to 5.7 ml DMEM with GlutaMAX (serum-free media) in white-capped conical tubes (Sterilin) and incubated for 5 min at room temperature. A total of 40 ug DNA was added (20 ug of each plasmid), the mixture was incubated for a further 30 min at room temperature and then added dropwise to the cells. Flasks were incubated for 40-48 hrs at 37ºC in a 5% CO_2 humidified incubator and the medium was changed 6 hrs post-transfection (cDMEM).

4.2.4.3 Extraction of pseudovirions

Pseudovirions were harvested 40-48 hrs post-transfection. Cells were collected by trypsinisation with 0.05% Trypsin-EDTA (Gibco) and inactivated by the addition of cDMEM. The cells were transferred to a conical-bottomed polystyrene Sterilin tube (as pseudovirions adsorb non-specifically to polypropylene tubes), counted and centrifuged at 1200 rpm x 8 min. The pellet was washed with 0.5ml DPBS (Invitrogen) and resuspended in 1.5 pellet volumes of DPBS-Mg (DPBS with an additional 9.5 mM $MgCl_2$) to achieve a high cell density of >100 x 10^6 cells/ml.

10% Brij-58 (Sigma) was added to the resuspended pellet to a final concentration of 0.5% (w/v) and both Benzonase (Sigma) and Plasmid-Safe™ ATP-dependent DNase (Epicentre) were added to 0.5% (v/v) and 0.2% (v/v) respectively. Using Chris Buck's "Improved Maturation of HPV and Polyomavirus" protocol, sterile ammonium sulphate (1M, pH 9.0) was added to a final concentration of 25 mM to promote the formation of intermolecular L1 disulphide bonds. The mixture was incubated at 37ºC for 15 min to allow lysis and then transferred to the preferred temperature for pseudovirion maturation overnight (25ºC for HPV-16 and 18, 37ºC for HPV-45 and 52).

The matured lysate was chilled on ice for 5 min and the final NaCl concentration of the lysate was adjusted to 850 mM and incubating on ice for a further 10 min. The lysate was clarified by centrifuging 3000 x g for 10 min at 4ºC. The supernatant was collected and the pellet was re-extracted by resuspending in an equal pellet volume of high salt DPBS (0.8M NaCl) and re-centrifuging. The supernatants were pooled, re-centrifuged and transferred into white-capped polystyrene tubes and kept on ice.

4.2.4.4 Purification of pseudovirions

PsV are purified by Optiprep density gradient centrifugation. Optiprep (60% w/v iodixanol solution; Sigma) was diluted in DPBS to a 46% (w/v) Optiprep stock solution, and supplemented with 0.625M NaCl to a final concentration of 0.8M NaCl, $CaCl_2$ to 0.9mM, $MgCl_2$ to 0.5mM and KCl to

2.1mM. High salt DPBS (0.8M NaCl) was used to dilute the stock solution to 27%, 33% and 39% Optiprep, and the 3-step gradient was prepared by underlaying the Optiprep dilutions (27 – 39%) in 1.5ml steps in thin wall 5ml polyallomer ultracentrifuge tubes (Beckman). The gradient was left to diffuse at room temperature for 4 hrs. Double-clarified cell supernatant was layered onto the linearized Optiprep gradient and centrifuged in a Beckman SW55ti rotor at 50,000 rpm (234,000 x g) for 3.5 hrs at 16ºC. The bottom of the tube was punctured with a syringe needle and fractions were collected in white-capped polystyrene tubes: the first fraction was ~0.75 ml, fraction 2-11 was ~0.25 ml each and fraction 12 contained the remainder of the gradient.

The protocol for screening fractions was modified to detect the presence of HPV L1, the major protein present in the capsid (Buck *et al*., 2008), using HPV type-specific anti-L1 dot blots. Each fraction was spotted onto nitrocellulose membrane (0.5 µl) and Cervarix (HPV-16 L1), *E. coli*-produced His-tagged HPV-16 L2, or the clarified HPV-16, 18, 45 or 52 supernatant initially loaded onto the gradient was used as positive controls.

The membranes were blocked in blocking buffer for 30 min at room temperature and then probed overnight at room temperature with an appropriate primary anti-L1 antibody diluted in blocking buffer. CamVir1 (1:5000; Abcam) was used to detect HPV-16. In addition, rabbit anti HPV-16 L2 sera was available and used to detect L2 in the HPV-16 fractions (1:2000). The H16.I23, H45.N5, H52.C1 and H52.D11 MAb kindly provided by Dr Neil Christensen were used to detect HPV-18, 45 and 52 respectively (1:2000; Christensen *et al*., 1996). Membranes were probed with 1:10,000 secondary antibody (goat anti-mouse IgG conjugated to alkaline phosphatase or goat anti-rabbit alkaline phosphatase conjugate; Sigma), washed and developed as previously described (Chapter 2, Section 2.2.8). Peak fractions containing a high concentration of L1 were pooled in polystyrene tubes and stored at -70ºC for titration.

4.2.4.5 Electron microscopy of pseudovirions

Purified HPV PsV were analyzed using electron microscopy. The PsV's (1:1000) were trapped on glow-discharged carbon-coated copper grids, stained with 2% uranyl acetate and viewed using a Zeiss EM 912 CRYO EFTEM.

4.2.4.6 Pseudovirion titration

The PsV titrations and neutralisation assays were based on the "Papillomavirus Neutralisation Assay" protocol, with the exception that no NAb were included in the titration. PsV stocks were titrated prior to the neutralisation assays in order to determine the minimum amount of PsV required for a robust signal in the SEAP assay.

HEK293TT cells were grown in cDMEM-Ab to 70-80% confluence, collected as described, washed with DPBS and diluted to 3.0 x 10^5 cells/ml in neutralisation media (High glucose cDMEM with HEPES and without phenol red or sodium pyruvate, supplemented with 10% fetal calf serum; Gibco). Cells were pre-plated into 96-well tissue culture treated plates (Corning Costar) with 100µl cell suspension in each internal well and 150µl DMEM with phenol red in the external wells to avoid evaporation from the inner wells. Cells were incubated for 3-4 hrs at 37ºC before the addition of the PsVs.

Serial dilutions of PsVs were prepared in neutralisation media (doubling dilutions from 1:250 to 1:64000) in non-treated sterile 96-well polystyrene plates (Nunc) and tested in triplicate. The PsV dilutions were added to the pre-plated cells (100µl / well) as outlined in the Schiller protocol, and each plate contained 6 negative control wells with no pseudovirions (cell control). Plates were incubated for 72 hrs at 37ºC in a humidified CO_2 incubator.

SEAP activity was detected using the Great EscAPe™ SEAP Chemiluminescence Kit 2.0 (Clontech Laboratories, Inc.) according to manual instructions, except volumes were adjusted to 0.6 volumes of those given in the manufacturer's protocol (as done in the revised Schiller protocol).

Supernatant (125µl) was transferred into sterile untreated 96-well polysterene plates (Nunc) and centrifuged at 1000 x g for 5 min. Clarified supernatant (15µl) was transferred into a white 96-well Optiplate (96F white maxisorb luminometer plates; Nunc), 45µl 1x dilution buffer was added to each well and the plate was incubated at 65°C for 30 min. Plates were chilled for 5 min on ice and then 60µl substrate was added per well and incubated at room temperature for 20 min. SEAP production was detected using a microplate luminometer (Digene DML 2000). The PsV dilution chosen for the neutralisation assay was one that used the minimum amount of PsVs occurring within the linear range of the titration curve. As the HPV-52 titre was very low, it was re-titred from 1:125 to 1:4000.

4.2.4.7 Pseudovirion neutralisation assay

An *in vitro* neutralisation assay was used to detect HPV-specific antibody responses in mouse sera and to determine endpoint neutralisation titres.

Controls included:

(a) Cell control (negative infection control): Cells were incubated with neutralisation media only (no sera or pseudovirions) to give a background reading of the cell culture supernatant. The luminescent values associated with this control represented 0% PsV neutralisation.

(b) PsV control (positive PsV infection control): PsVs were pre-incubated in neutralisation buffer prior to cell infection. The values associated with this control represented 100% PsV neutralisation.

(c) MAb or antisera known to neutralise the HPV-type PsV used in the assay (positive neutralisation assay control): PsV's were pre-incubated with 6 dilutions which should span the pre-determined neutralisation titre (0-100% neutralisation).

(d) Pre-bleeds: PsV's were pre-incubated with pooled mouse pre-bleeds (negative control).

The NAb positive controls (Table 4) were titrated prior to the test sera neutralisation assay in order to determine the neutralisation dilution range to be used in the PsV neutralisation assays. The HPV-16, 45 and 52 neutralisation controls were H16.V5, H45.N5, H52.C1 and H52.D11 MAb. The HPV-18 control was rabbit anti-Cervarix sera from our laboratory.

Table 4: HPV type-specific neutralising antibodies

Positive control antibody	HPV type neutralised	Fold dilution	Dilution range
Mouse H16.V5 ascites	HPV-16	10-fold	$2x10^2 – 2x10^7$
Rabbit anti-Cervarix sera	HPV-18	4-fold	50 – 51200
Mouse H45.N5 ascites	HPV-45	4-fold	800 – 819200
Mouse H52.C1 supernatant	HPV-52	10-fold	$2x10^2 – 2x10^7$
Mouse H52.D11 supernatant	HPV-52	10-fold	$2x10^2 – 2x10^7$

Sera from mice immunized with plant-produced HPV-16 chimaera candidate vaccines were pooled (10 mice/vaccine) and tested for neutralisation of HPV-16, as well as HPV-18, 45 and 52. Pooled vaccine sera was diluted 4-fold in triplicate in the range 1:50 to 1:12800. Pre-bleeds were also pooled and tested in triplicate as a negative control at the lowest dilution of 1:50. Serial dilutions of sera were prepared in sterile non-treated 96-well tissue culture plates (1:10 to 1:2560).

PsVs were diluted in neutralisation buffer to the concentration pre-determined in the titration assay. In another untreated 96-well plate, 100µl diluted PsVs were added to each well and 25µl of diluted sera (or neutralisation buffer for the PsV control wells) were added to the triplicate wells, resulting in a further 1:5 dilution of pre-diluted sera. The PsVs and sera were incubated at 4°C for 1hr to allow for the neutralisation of infectious PsVs, and then 100µl were added to each well in the pre-plated HEK-293TT plate (neutralisation buffer for the cell control wells). The plates were incubated for a further 72 hrs in a 37°C humidified CO_2 incubator.

The supernatant was harvested as described above and assayed for the presence of SEAP. The neutralisation titre was stated as the reciprocal of the maximum serum dilution which reduces SEAP activity by at least 50% in comparison to the control sample not pre-incubated with serum.

4.3 Results

4.3.1 Humoral immune response against HPV-16 L1

The detection of antibodies elicited against HPV-16 L1 was done by direct ELISA, using insect cell-expressed HPV-16 L1 as the coating antigen (Figure 3). The anti-L1 titres were expressed as the reciprocal of the maximum serum dilution containing higher absorbance readings than that of the corresponding pre-bleed serum at 1:50.

No anti-L1 response was detected for the L1/L2(56-81) chimaera and the negative control vaccine (V2 and V5; Figure 3A) as well as the vaccine pre-bleeds (Figure 3C). In comparison, the ELISA MAbs (H16.V5, CamVir1, Figure 3B) and the plant-derived L1 positive controls (V4, Figure 3A) showed a good response and both the plant-derived L1/L2(17-36) and L1/L2(108-120) chimaeras elicited anti-L1 titres of 200 and 12800 respectively (V3 and V1, Figure 3A). Although HPV-16 L1 elicited the highest anti-L1 titres (12800-51200), L1/L2(108-120) showed a similar response (V4 and V1 respectively, Figure 3A), suggesting the insertion of the L2 aa 108-120 epitope had less of an effect on L1 immunogenicity in comparison to the other chimaeras. Furthermore, the L1/L2(108-120) and HPV-16 L1 anti-L1 response was statistically significant from their corresponding pre-bleeds and the NSs-infiltrated plant extract (p = 0.01).

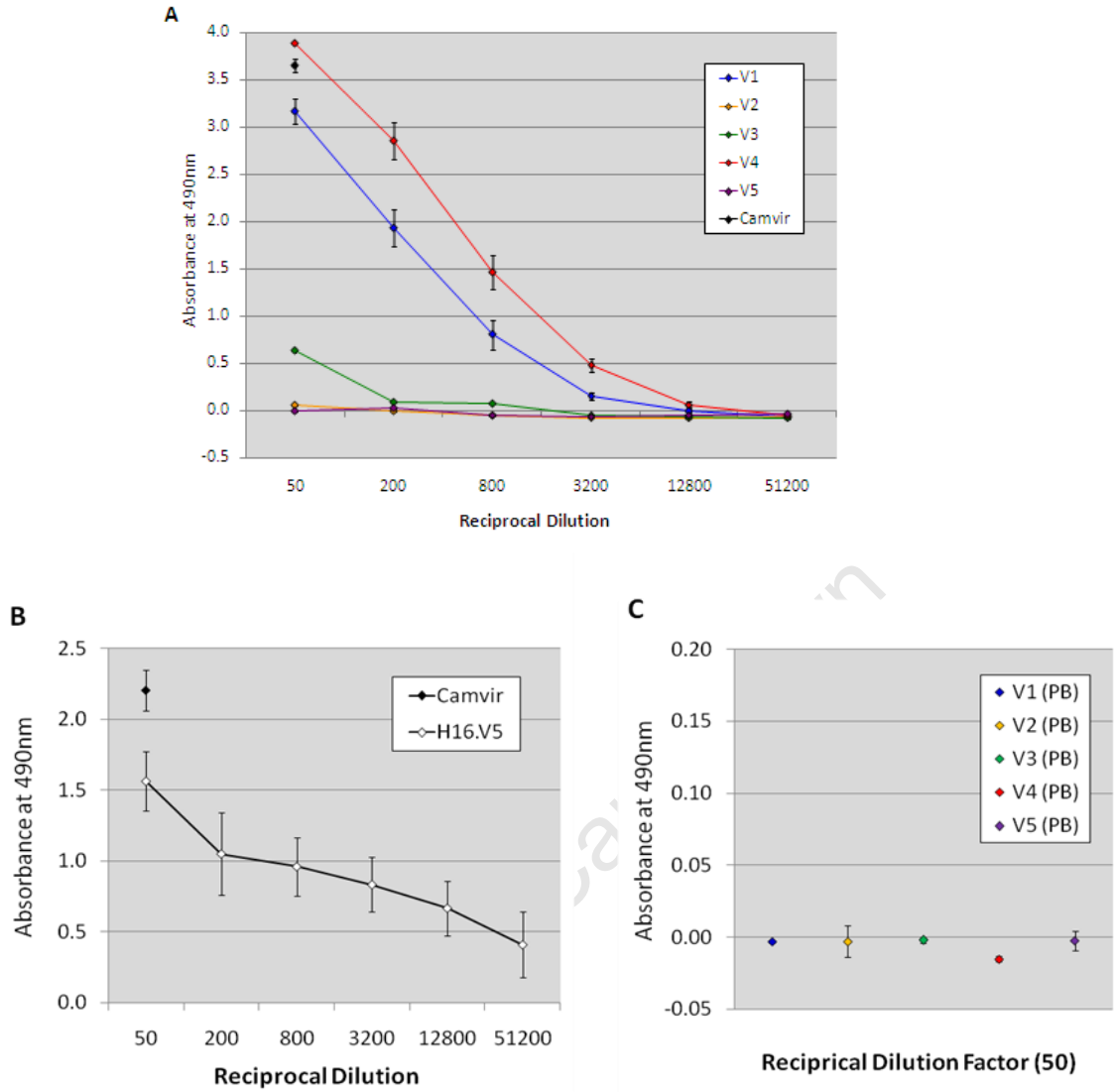

Figure 3: Direct ELISA of mouse sera using insect cell-produced HPV-16 L1 as the coating antigen. V1 = L1/L2(108-120), V2 = L1/L2(56-81), V3 = L1/L2(17-36), V4 = HPV L1 (+ vaccine control), V5 = plant extract (- vaccine control). A) Titration of the mouse antisera for all the vaccines. B) Graph showing the values obtained for the ELISA positive control MAbs H16.V5 and CamVir1. C) Vaccine pre-bleed absorbance values at 1:50 dilution. Markers represent the mean value of triplicate samples and error bars indicate the standard deviation.

4.3.2 Humoral immune response against the HPV-16 L2 epitopes

The anti-L2 response against the *E. coli*-produced His-tagged HPV-16 L2 protein was determined using western blotting. Individual mouse sera were pooled for each of the vaccines and analysed for anti-L2 responses (Figure 4).

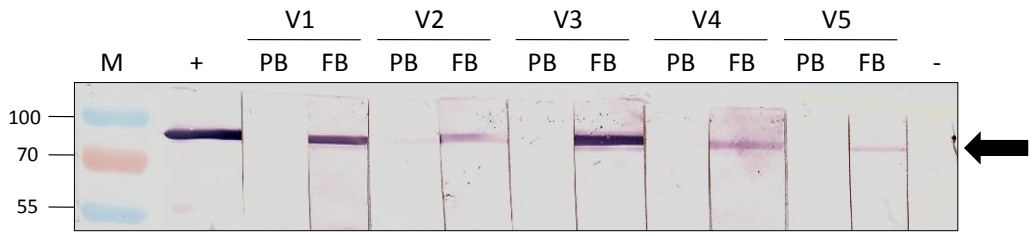

Figure 4: Western blot detection of *E. coli*-expressed His-tagged HPV-16 L2 by mouse sera at a dilution of 1:100. M = protein marker with the protein size in kDa. V1 = L1/L2(108-120), V2 = L1/L2(56-81), V3 = L1/L2(17-36), V4 = HPV L1 (+ vaccine control), V5 = plant extract (- vaccine control). PB = pre-bleed sera. FB = final bleed sera. For the western blot controls: +ve = mouse anti-His (1:2000; Serotec), -ve = no primary antibody. The black arrow indicates L2 (~80 kDa).

A non-specific band similar to the ~80 kDa L2 band was detected in both the antisera from the negative vaccine control (V5; plant extract) and the L1 vaccine control (V4; plant-expressed HPV-16 L1) which serves as an additional negative L2 control in this experiment (Figure 4). All chimaera vaccines (V1-3) appeared to give an anti-L2 response, as strong L2 bands were detected using the chimaera antisera (Figure 4). However, only the L1/L2(108-120) and L1/L2(17-36) chimaeras (V1 and V3 respectively) gave a definitive anti-L2 response, with L2 bands >2X intensity of HPV-16 L1 (V4).

4.3.3 Neutralisation assays

4.3.3.1 Plasmid analysis

The identity of the pYSEAP and the HPV-16, 18, 45 and 52 L1/L2 pSheLL plasmids was confirmed using restriction enzyme digestion and sequencing (data not shown).

4.3.3.2 Optiprep purification and HPV PsV detection in purified fractions

HPV PsVs were purified from the clarified cell supernatant by density gradient ultracentrifugation on a 27-39% Optiprep linear gradient. A light grey band was faintly visible a third of the way up from the gradient and the fractions were collected from the bottom of the tube (Figure 5).

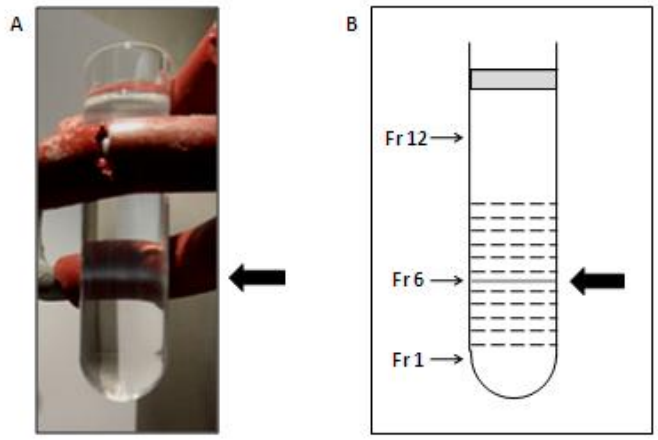

Figure 5: Purified HEK293TT-produced HPV PsVs. The arrow indicates the purified HPV PsV. A) Photograph of an Optiprep density gradient after ultracentrifugation of clarified HPV-45 PsV. All HPV PsV types showed similar banding. B) Schematic diagram of the Optiprep fractionation.

Fractions were screened for the presence of PsVs using HPV type-specific anti-L1 dot blots (Figure 6). CamVir1 and rabbit antisera against HPV-16 L2 was used to detect HPV-16 L1 and L2 (Figure 6A), using Cervarix and *E. coli*-produced His-tagged HPV-16 L2 as controls. The H16.I23, H45.N5, H52.C1 and H52.D11 MAb were used to detect HPV-18, 45 and 52 respectively (Figure 6B-D), using the initial clarified cell supernatant as the HPV type-specific control.

HPV-16 was detected in fraction 3-5 using H16.V5 and weakly detected with the HPV-16 L2 antisera, as the L2 protein is located internally to the L1 capsid surface in co-assembled L1/L2 VLPs (Buck *et al*., 2008). HPV-18, 45 and 52 L1 was strongly detected in fractions 5-7, 4-6 and 6-10 respectively. PsV fractions were pooled, examined by electron microscopy and used in the neutralisation assays.

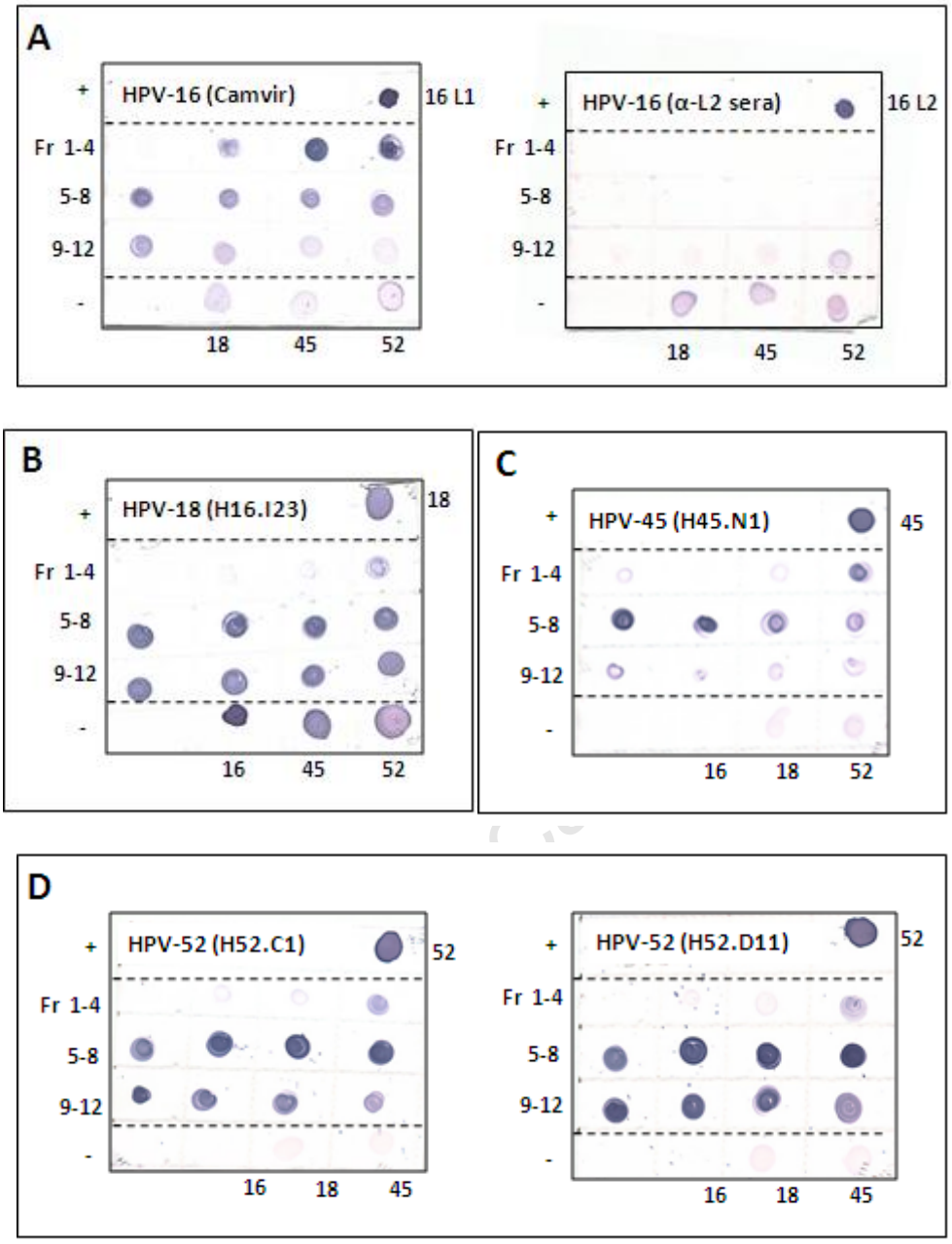

Figure 6: Dot blot detection of purified HEK293TT-produced HPV PsVs in the Optiprep fractions. A) HPV-16 PsVs were detected with anti-L1 MAb CamVir1 and anti-L2 MAb rabbit sera. B) HPV-18 PsVs were detected with H16.I23. C) HPV-45 PsVs were detected with H45.N5. D) HPV-52 PsVs were detected with H52.C1 and H52.D1. Fractions 1-12 were tested, using the clarified PsV supernatant layered onto the gradient as the +ve control (top right corner of each blot), and the PsV supernatant of the other PsV types as –ve controls (bottom of each blot). Fractions containing a high concentration of PsV were pooled and then titrated.

4.3.3.3 Electron microscopy analysis

The pooled PsV samples were examined by transmission electron microscopy to determine their assembly, morphology and purification (Figure 7). All HPV types assembled into spherical PsVs (55 nm). HPV-45 PsVs appeared to exist exclusively as fully-assembled PsV particles (Figure 7D). HPV-16 and 18 PsVs were predominantly assembled, although some capsomeres and aggregates were visible (Figure 7A-B). HPV-52 PsVs (Figure 7D) contained a large proportion of capsomere aggregates and partial PsVs, possibly as a result of low HPV-52 L1 and L2 expression in the HEK293TT cells.

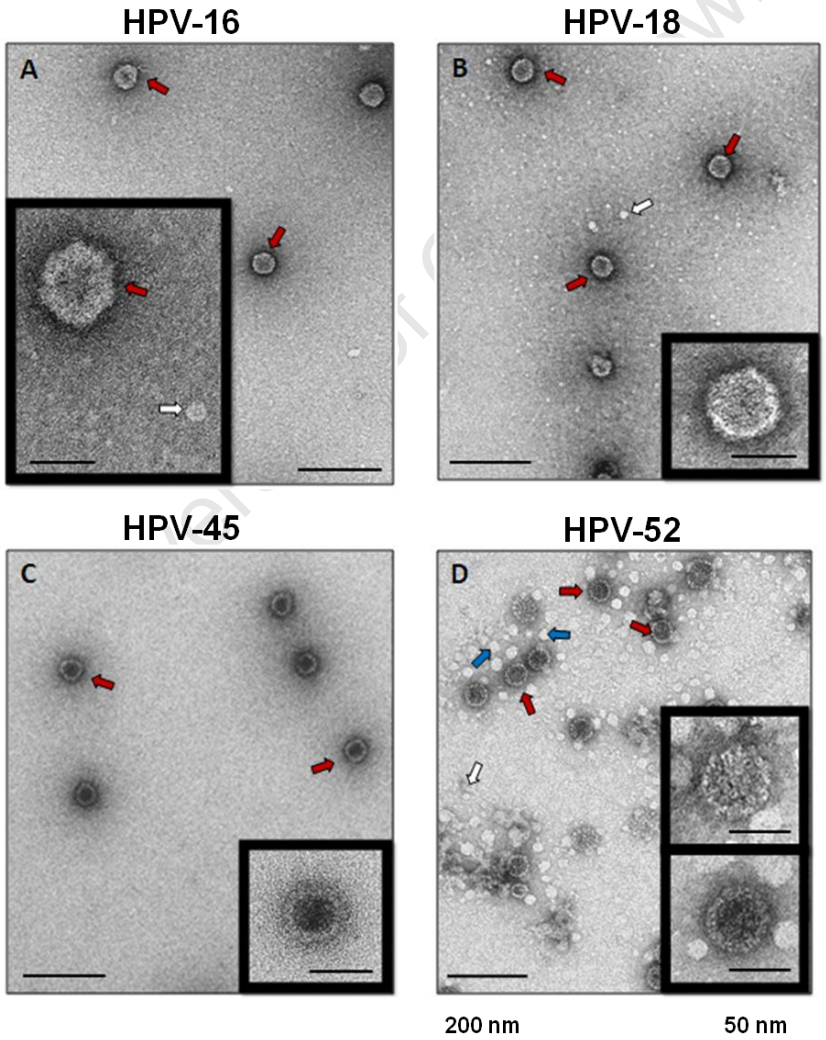

Figure 7: Electron microscopy of purified HEK293TT-produced HPV PsVs viewed on a Zeiss EM 912 CRYO EFTEM. A) HPV-16, B) HPV-18, C) HPV-45 and D) HPV-52 PsV. The red arrows indicate HPV PsV (~55 nm), the blue arrows indicate capsomere aggregates (20-25 nm), and the white arrows indicate capsomeres (~10 nm).

4.3.3.4 HPV PsV titration

The purified PsVs were titrated (Figure 8) to determine the PsV dilution to be used for the neutralisation assays. The dilution used was the minimum amount of PsVs giving a robust signal within the linear range of the titration curve.

For HPV-16 and 18 PsVs, the linear range of the titration curve occurred between dilutions 1:250 to 1:1000 (Figure 8A), and thus 1:500 was chosen for the neutralisation assays. HPV-45 PsVs had the highest titre, with the linear range occurred between dilutions of 1:500 and 1:2000 (Figure 8A), thus 1:1000 was chosen for further work. HPV-52 PsVs had to be re-titred using lower dilutions (poor titre yield has also been reported by Schiller's group). The linear range occurred between dilutions 1:125 to 1:250 (Figure 8B), and a 1:200 dilution was used in the HPV-52 neutralisation assay.

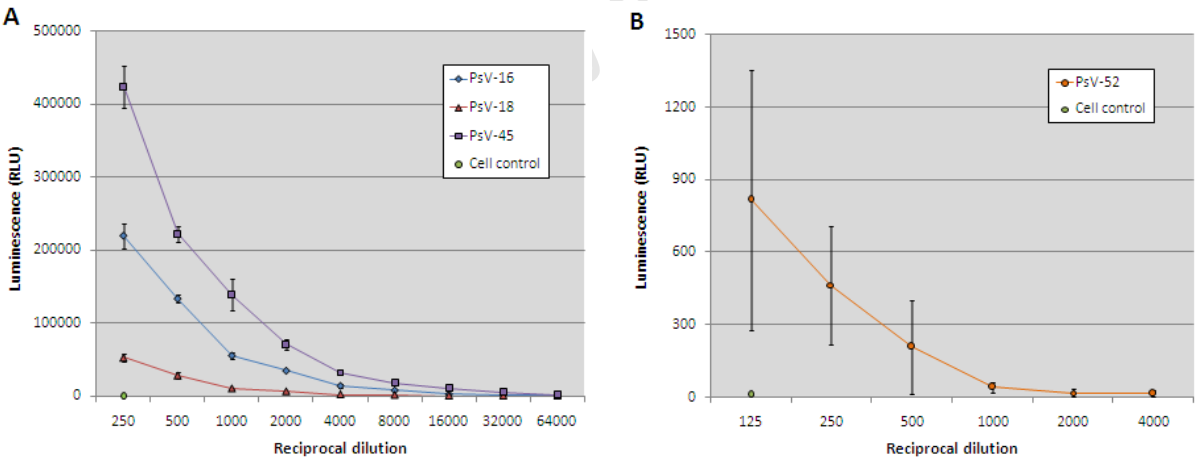

Figure 8: HPV PsV titration curves. A) High-yielding HPV-16, 18 and 45 PsV titrations. B) Low-yielding HPV-52 PsV titration. The error bars indicate the standard deviation.

4.3.3.5 Titration of the positive control neutralising antibodies

The NAb positive controls were tested prior to the neutralisation assays with the mouse antisera, in order to check their neutralising ability and to

determine a suitable dilution range. All positive control antibodies were neutralising and showed a linear relationship within the dilution range tested (Table 5).

Table 5: Titration of the positive control neutralising antibodies

HPV PsV type	Positive control	Dilution range	PsV neutralisation (%)
HPV-16	H16.V5	$2x10^2 – 2x10^7$	19 – 100
HPV-18	anti-Cervarix sera	50 – 51200	34 – 99
HPV-45	H45.N5	800 – 819200	29 – 100
HPV-52	H52.C1	$2x10^2 – 2x10^7$	0 – 98
	H52.D11	$2x10^2 – 2x10^7$	0 – 98

4.3.3.6 HPV PsV neutralisation assays

Sera from mice immunized with plant-produced HPV-16 L1 and L1/L2 chimaeras were tested for homologous neutralisation of HPV-16 PsVs and heterologous cross-protection against HPV-18, 45 and 52 PsVs (Figure 9-12). All positive control NAbs successfully neutralised the HPV-16, 18, 45 and 52 PsVs (Figure 9-12F), demonstrating that the neutralisation assay results were valid. The neutralisation titre was defined as the highest dilution of serum which reduces SEAP activity by >50% in comparison to the control sample, which was not treated with serum.

<u>HPV-16</u>

The results from the HPV-16 PsV neutralisation assays are shown in Figure 9. Plant-derived HPV-16 L1 sera (V4; Figure 9D) mimicked the H16.V5 positive control (Figure 9F) and strongly neutralised HPV-16 PsV, followed by L1/L2(108-120) with a similar neutralisation curve (V1; Figure 9A). Both L1/L2(56-81) and L1/L2(17-36) did not appear to elicit HPV-16 NAb (V2-3; Figure 9B-C) showing similar neutralisation curves to the negative control (V5; Figure 9E).

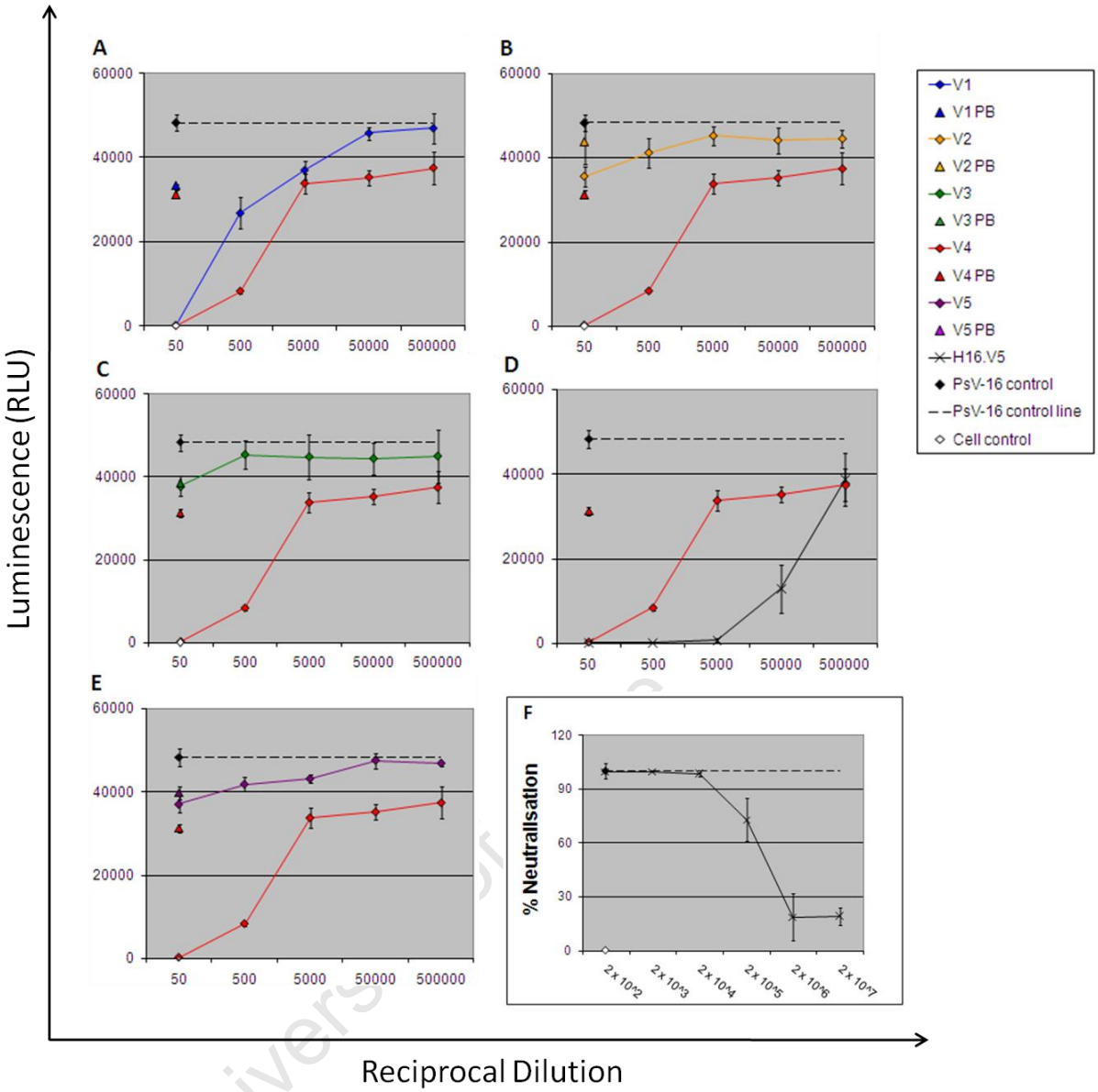

Figure 9: HPV-16 PsV neutralisation assay. Pooled sera from mice vaccinated with V1-V5 were tested for their ability to neutralise HPV-16 PsVs. A) V1 = L1/L2(108-120), B) V2 = L1/L2(56-81), C) V3 = L1/L2(17-36), D) V4 = HPV-16 L1 (+ve vaccine control), E) V5 = NSs-infiltrated plant extract (-ve vaccine control). F) H16.V5 = +ve neutralisation control. Cell control = -ve infection / SEAP expression control. PsV control = +ve infection / SEAP expression control. Samples were assayed in triplicate and error bars indicate the standard deviation.

HPV-18

The antisera from all the vaccines did not neutralise HPV-18 PsV (Figure 10). The L1/L2(56-81) and L1/L2(17-36) chimaeras (V2-3, Figure 10B-C) produced neutralisation curves similar to the type-specific HPV-16 L1 vaccine and the

negative control (V4-5, Figure 10D-E). L1/L2(108-120) appeared to have some neutralising activity, with reciprocal sera dilutions of <800 reducing luminescent readings below that of the pre-bleed and the unneutralised HPV-18 PsV control (V1; Figure 10A). However, the chimaera did not reduce SEAP levels by >50%.

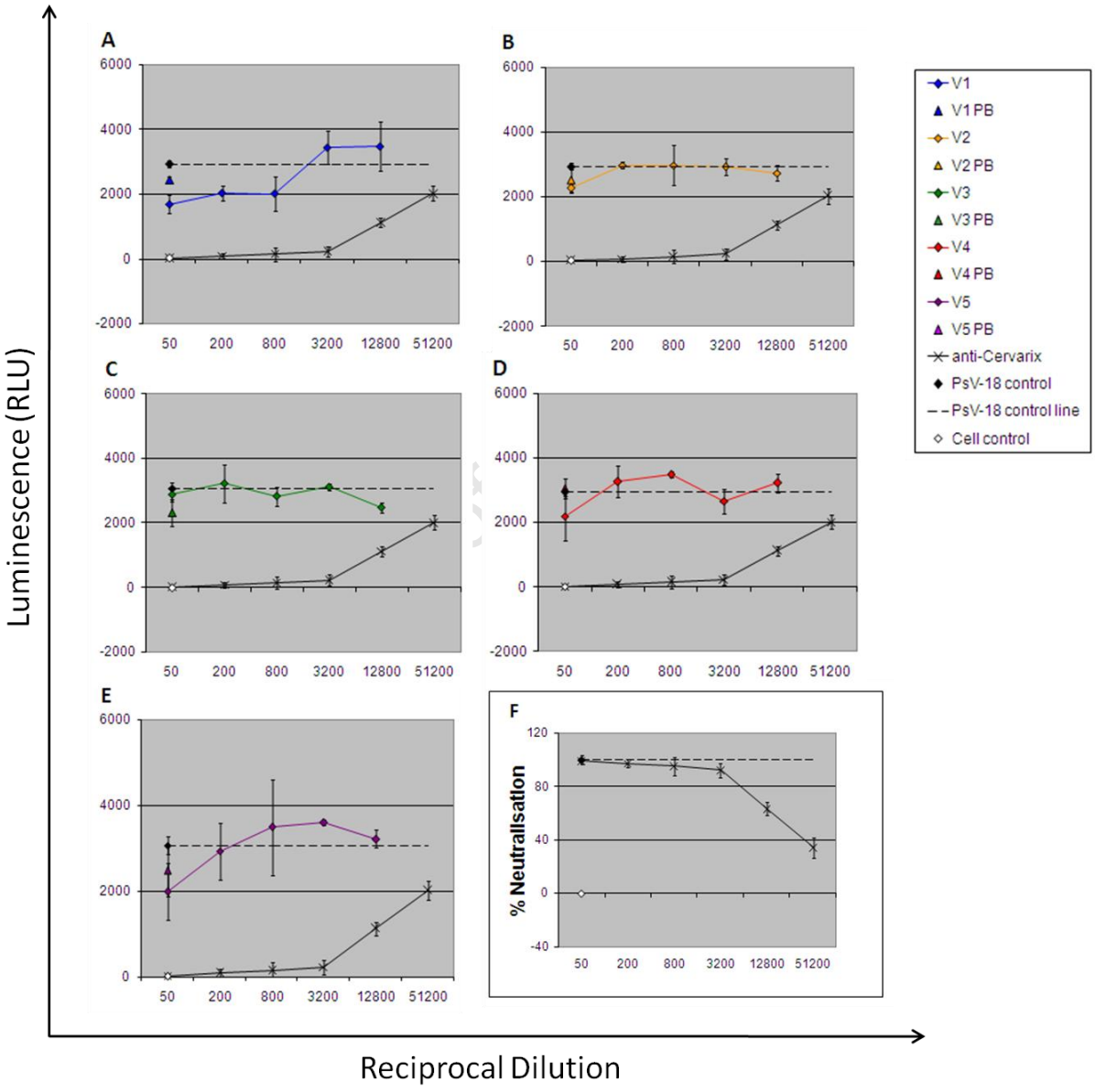

Figure 10: HPV-18 PsV neutralisation assay. A) V1 = L1/L2(108-120), B) V2 = L1/L2(56-81), C) V3 = L1/L2(17-36), D) V4 = HPV-16 L1, E) V5 = NSs-infiltrated plant extract (-ve vaccine control). F) Rabbit anti-Cervarix sera = +ve neutralisation control.

HPV-45

The results from the HPV-45 PsV neutralisation assay (Figure 11) suggest that none of the L1/L2 chimaera vaccines (V1, V2 and V3; Figure 11A-C) elicited significant titres of HPV-45 NAb, with neutralising curves similar to HPV-16 L1 and the negative vaccine control (V4-5; Figure 11D-E).

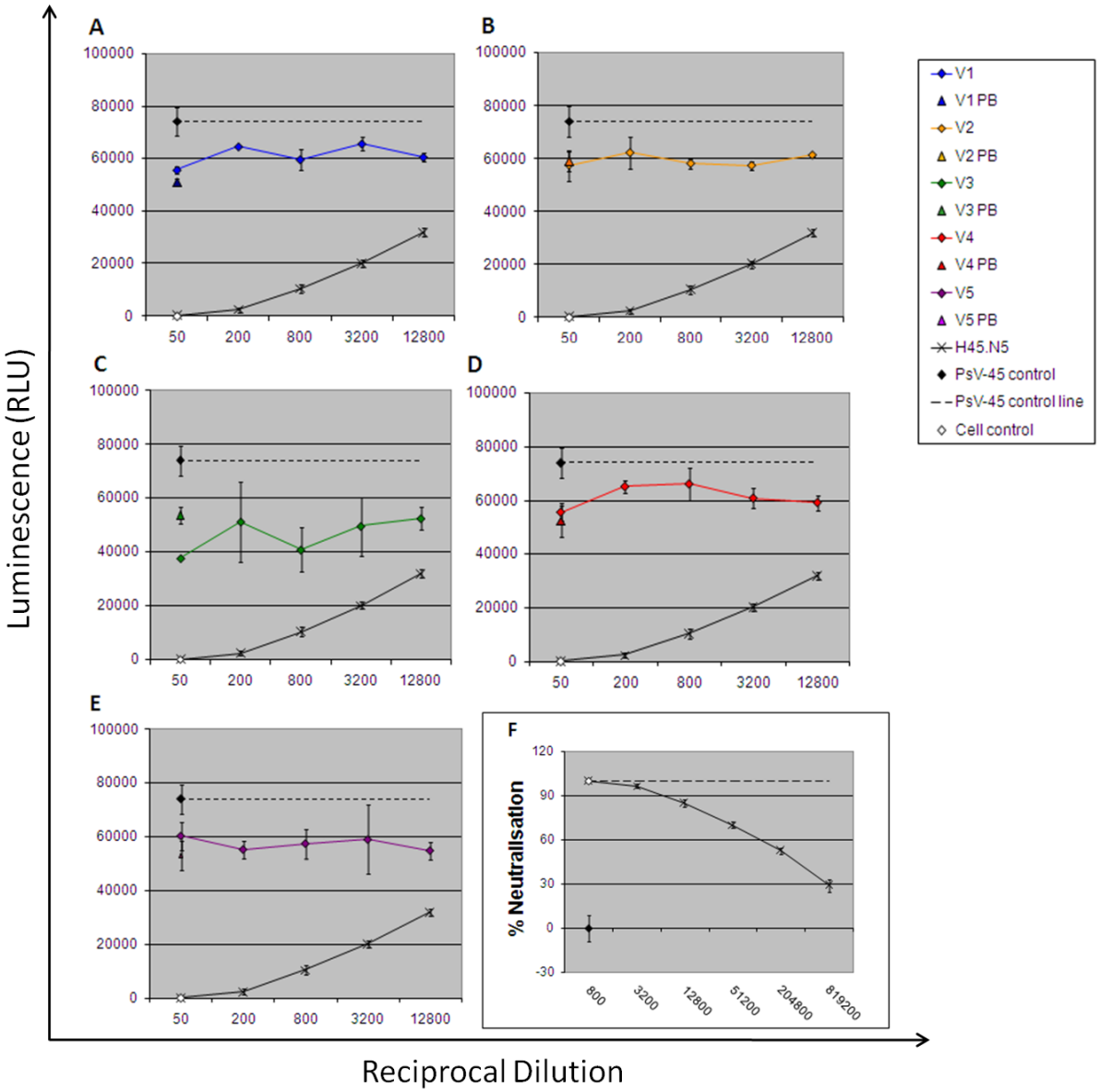

Figure 11: HPV-45 PsV neutralisation assay. A) V1 = L1/L2(108-120), B) V2 = L1/L2(56-81), C) V3 = L1/L2(17-36), D) V4 = HPV-16 L1, E) V5 = NSs-infiltrated plant extract (-ve vaccine control). F) H45.N5 = +ve neutralisation control.

HPV-52

The HPV-52 PsV neutralisation assays (Figure 12) provide evidence that L1/L2(56-81) sera did not neutralise HPV-52 (L2; Figure 12C), as seen for HPV-16 L1 and the negative control sera (V4-5; Figure 12D-E). L1/L2(108-120) and L1/L2(17-36) chimaera vaccines appeared to have some neutralising activity at low reciprocal dilutions (50-200), reducing SEAP levels by >50% in comparison to the unneutralised HPV-52 PsV control (V1 and V3; Figure 12A and C).

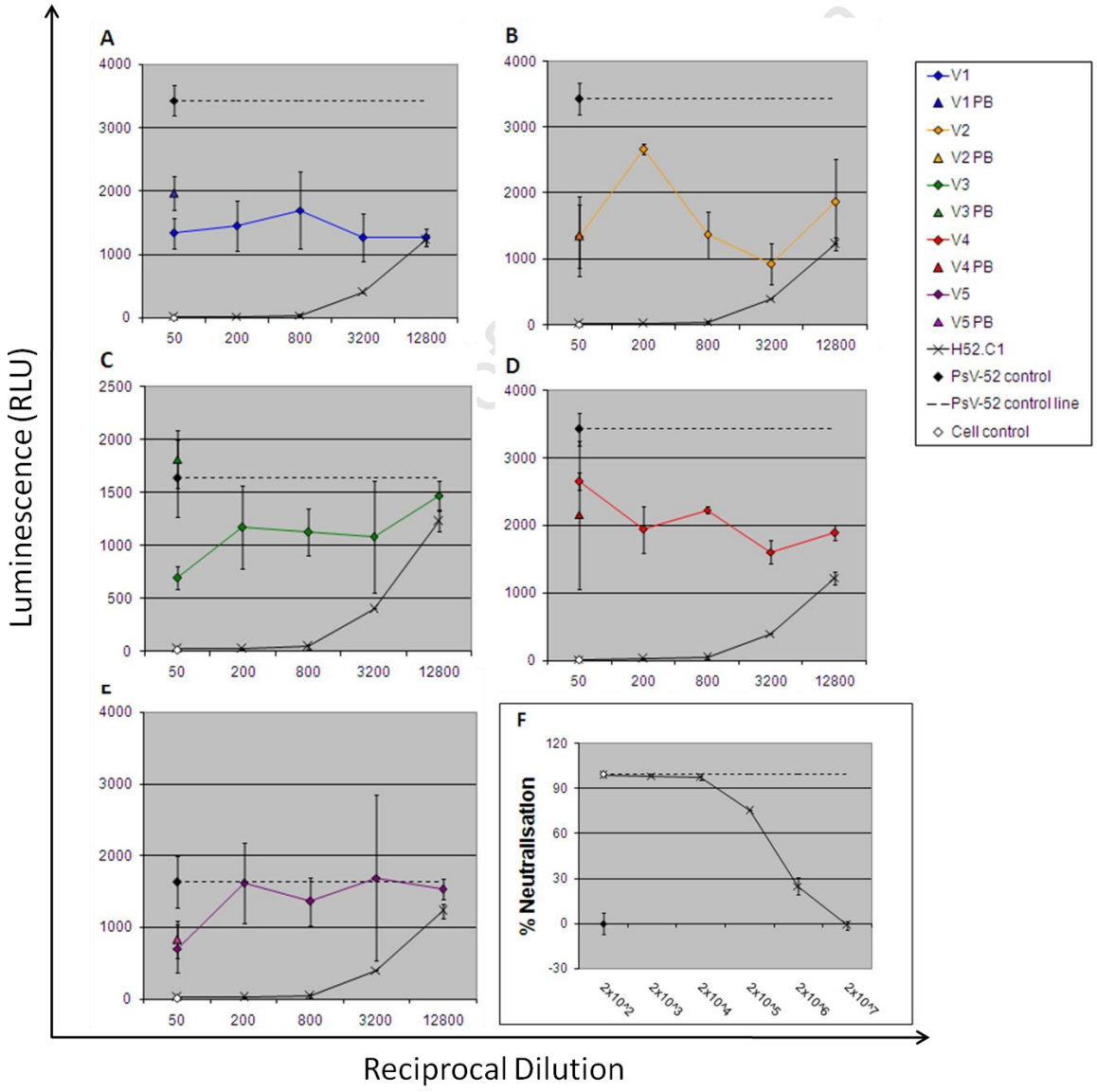

Figure 12: HPV-52 PsV neutralisation assay. A) V1 = L1/L2(108-120), B) V2 = L1/L2(56-81), C) V3 = L1/L2(17-36), D) V4 = HPV-16 L1, E) V5 = NSs-infiltrated plant extract (-ve vaccine control). F) H52.C1 = +ve neutralisation control.

Although the assay was successful, as shown by the H52.C1 NAb control (Figure 12F), there was a great deal more variation between triplicates samples and trend lines were difficult to establish. This may be attributed to the partial purification and low concentration of HPV-52 PsVs which may have exaggerated small differences between replicates. The values for the HPV-52 PsV infection control differ between vaccines as V1, V2 and V4 (Figure 12A-B and D) were analyzed on a different plate from V3, V5 and H52.C1 (Figure 12C and E-F). Time constraints prevented this assay from being repeated.

Table 6 summarizes the HPV-16, 18, 45 and 52 PsV neutralisation antibody titres elicited by the plant-derived vaccines. L1/L2(108-120) elicited homologous HPV-16 NAb and the antisera cross-neutralised heterologous HPV-52 PsV, suggesting this vaccine has the most potential for protection. L1/L2(17-36) chimaeras elicited low levels of cross-neutralising HPV-52 NAb, but homologous HPV-16 NAb were not detected, suggesting the immunogenicity against HPV-16 L1 may be compromised. L1/L2(56-81) did not elicit NAb and does not appear to have potential as a prophylactic vaccine. None of the HPV vaccines elicited cross-neutralising antibodies against phylogenically-related HPV types 18 and 45.

Table 6: Summary of the neutralisation titres for plant-derived L1 and the L1/L2 chimaera candidate vaccines

		PsV neutralisation assay titres*			
Vaccine	**Chimaera**	**HPV-16**	**HPV-18**	**HPV-45**	**HPV-52**
V1	L1/L2(108-120)	50-500	0-50	0-50	50-200
V2	L1/L2(36-58)	0-50	0-50	0-50	0-50
V3	L1/L2(17-36)	0-50	0-50	0-50	50-200
V4	HPV-16 L1	500-5000	0-50	0-50	0-50
V5	Plant extract	0-50	0-50	0-50	0-50
+ control	H16.V5	$2x10^5 – 2x10^6$			
	α-CamVir1		12800-51200		
	H45.N5			3200-12800	
	H52.C1				$2x10^4 – 2x10^5$
	H52.D11				$2x10^5 – 2x10^6$

4.3.4 Overview of vaccine immunogenicity

The structural assembly (Chapter 3, Section 3.3.3), the anti-L1 and L2 humoral responses and the HPV-type NAb detected in the L1/L2 chimaera antisera are summarized in Table 7. Assembly into VLPs appears to be associated with higher anti-L1 and HPV-16 PsV neutralisation titres, suggesting assembly is associated with L1 immunogenicity.

Table 7: Antibody responses for the L1 and L1/L2 chimaeric vaccines

Vaccine	Plant-expressed antigen	TEM structure*	Anti-L1 response**	Anti-L1 titres	Anti-L2 response***	HPV-16/18/45/52 neutralisation
V1	L1/L2(108-120)	VLPs	Y	12800	Y	HPV-16/52
V2	L1/L2(56-81)	C / CA	N	0-50	N	None
V3	L1/L2(17-36)	CA / VLPs	Y	200	Y	HPV-52
V4	HPV-16 L1 (+)	VLPs	Y	>12800	N	HPV-16
V5	Plant extract (-)	N/A	N	0-50	N	None

* TEM antigen assembly: C = capsomeres, CA = capsomere aggregates, VLPs = virus-like particles.
** ELISA detection of anti-L1 antibodies. Y = yes, N = no.
*** Western blot detection of anti-L2 antibodies.

4.4 Discussion

Plant-derived HPV-16 L1 (Maclean *et al*., 2007; Fernández-San Millán *et al*., 2008) and L1-based chimaeras (Paz De la Rosa *et al*., 2009) assemble into immunogenic VLPs and elicit the production of neutralising antibodies (NAb), In this study, the immunogenicity of three plant-derived L1/L2 chimaeras containing cross-neutralising HPV-16 L2 aa 108-120, 56-81 or 17-36 epitopes in the h4 region of HPV-16 L1 were analysed. Mice were subcutaneously immunized with 10 µg of plant-derived antigen in Freund's incomplete adjuvant, and received 4 booster vaccinations within 7 weeks.

4.4.1 Humoral immune responses

Insect cell-expressed L1/L2 chimaeras with the L2 aa 108-120 substituted into the h4 region of L1 (Varsani *et al*., 2003a) or L2 aa 17-36, 18-31, 35-75, 69-81, 75-112, 108-120 and 115-154 inserted into the BPV-1 L1 DE surface

loop (aa 133-134) elicited anti-L1 and L2 responses in mice (Slupetzkey *et al*., 2007; Schellenbacher *et al*., 2009), suggesting L1/L2 chimaeras may be an effective strategy to broaden the protection of HPV prophylactic vaccines. As a result, the humoral anti-L1 and L2 responses elicited by the plant-derived L1/L2 chimaeras were analysed in this study, to determine if the L2 peptides are displayed and whether the L2 insertions compromise L1 immunogenicity.

The detection of L1 and L2 antibodies in mouse antisera was done by direct ELISA (Figure 3) and western blotting (Figure 4) respectively, using either insect cell-expressed HPV-16 L1 or *E. coli*-expressed His-tagged L2 antigen. Plant-derived HPV-16 L1 served as the anti-L1 positive control in the study and elicited the highest anti-L1 response, with titres of 12800 - 51200 (Figure 3A). These results are similar to other mouse immunogenicity studies using partially-purified plant-derived HPV-16 L1 VLPs (Titres = 20000 – 40960; Maclean *et al.*, 2007; Fernández-San Millán *et al.*, 2008).

The negative control vaccine (V5: NSs-infiltrated plant extract) and the vaccine pre-bleeds (V1-5 PB) did not give anti-L1 responses (Figure 3). However, antisera from the negative controls (V4-5, Figure 4) did detect the *E. coli*-expressed His-tagged HPV-16 L2 antigen, thus demonstrating the presence of non-specific antibodies in the sera which bound the His-tagged L2 protein. This is possibly due to the partial purification of antigens, which resulted in the vaccines containing contaminating plant proteins. Nevertheless, the negative control bands were less distinct than the bands for the L1/L2(108-120) and L1/L2(17-36) chimaeras, suggesting these L1/L2 chimaeras elicited an anti-L2 response.

L1/L2(108-120) assembled into distinctive cVLPs and was the most successful chimaera vaccine (Table 7), eliciting the highest anti-L1 response with titres of ~12800 (Figure 3A) and an anti-L2 response (Figure 4). Furthermore, only the L1/L2(108-120) and HPV-16 L1 antisera demonstrated significant anti-L1 responses (p = 0.01) in comparison to the pre-bleeds and the NSs-infiltrated plant extract (negative control). The insect cell-expressed L1/L2(108-120) chimaera analysed by Varsani *et al*. (2003a) elicited higher

anti-L1 titres (>204800) in comparison to the plant-derived chimaera, however a 10x higher dose was used (100 µg vs. 10 µg). Taken together, there is strong evidence that the L2 aa 108-120 peptide is effectively displayed on the surface of the L1 cVLPs.

The L1/L2(17-36) vaccine elicited a relatively weak anti-L1 response with titres of ~200 (Figure 3A) but elicited a strong anti-L2 response (Figure 4), suggesting that the L2 peptide is displayed on the surface of assembled L1. Similarly, fusion of a L2 aa 20-38 peptide to bacterial thioredoxin (Trx) elicited strong anti-L2 reponses in comparison to other Trx-L2 peptides comprising of aa 56-120 (Rubio *et al*., 2009) and the RG-1 MAb directed against the HPV-16 L2 aa 17-36 peptide has been shown to detect L2 in western blotting and ELISA (Gambhira *et al*., 2007b).

The L1/L2(56-81) capsomere vaccine did not elicit a detectable anti-L1 response at the lowest sera dilution 1:50 (Figure 3A) and the anti-L2 response was inconclusive (Figure 4), with both the anti-L1 and L2 responses similar to the vaccine pre-bleeds (V1-5 PB) and the negative controls (Figure 3-4). As a result, plant-derived L1/L2(56-81) do not appear to be immunogenic, unlike *E. coli*-expressed Trx-L2 fusion peptides (Rubio *et al*., 2009) and insect cell-expressed L1/L2 chimaeras containing similar L2 epitopes in the DE loop of BPV-1 L1 VLPs (Slupetzkey *et al*., 2007; Schellenbacher *et al*., 2009).

4.4.2 Pseudovirion neutralisation assays

The L1/L2 chimaeras, containing L2 epitopes aa 108-120, 56-81 and 17-36, were examined for their ability to elicit antibodies which neutralise HPV-16, 18, 45 and 52 PsVs. All the L2 epitopes analysed in this study have been shown to elicit antibodies which neutralise homologous HPV-16 and cross-neutralise HPV-52 (Kawana *et al*., 2003; Slupetzky *et al*., 2007; Kondo *et al*., 2007, 2008; Gambhira *et al*., 2007b; Schellenbacher *et al*., 2009). Additionally, L2 aa 56-81 cross-neutralises HPV-18 and L2 aa 17-36 cross-neutralises both HPV-18 and 45 (Gambhira *et al*., 2007b;

Kondo *et al*., 2007, 2008; Alphs *et al*., 2008; Schellenbacher *et al*., 2009; Rubio *et al*., 2009).

HPV-16 was chosen as HPV-16 L1 is the backbone of the chimaeric candidate vaccines and it causes the majority of cervical cancers, followed by phylogenically-related HPV-18 and HPV-45. HPV-16, 18 and 45 are associated with 48%, 23% and 10% of cervical cancers in Africa, and 61%, 10% and 6% of cervical cancers worldwide (de Sanjosé *et al*., 2010). Although HPV-52 is only ranked 5th in Africa (3%) and 6th worldwide (6%), HPV-52 has been shown to be highly prevalent in low and high-grade cervical lesions in South African women and thus HPV-52 cross-neutralisation is of local significance (Allan *et al*., 2008).

4.4.2.1 Homologous HPV-16 neutralisation

Plant-derived L1/L2(56-81) and L1/L2(17-36) did not elicit detectable HPV-16 NAb titres, giving results similar to the pre-bleeds and the NSs-infiltrated plant extract (Figure 9). Previous work has shown L1/L2 chimaeras containing HPV-16 L2 peptides aa 17-36, 18-38, 56-75 or 69-81 located in surface regions of BPV-1 or HPV-16 L1 elicted HPV-16 NAb (Slupetzkey *et al*., 2007; Kondo *et al*., 2008; Schellenbacher *et al*., 2009); however, the insertion sites differed from those used in this study and the chimaeras assembled into cVLPs. Furthermore, MAb directed against HPV-16 L2 aa 73-84 were found to be non-neutralising and did not neutralise HPV-16 PsV (Gambhira *et al*., 2007b), similar to the results obtained for the L1/L2(56-81) chimaera in this study.

In this study, only L1/L2(108-120) and HPV-16 L1 neutralised HPV-16 PsV in a similar manner to H16.V5 (positive neutralisation control), giving titres of 50-500 and 500-5000 respectively (Table 6). These results are consistent with other mouse immunogenicity studies using plant-derived HPV L1 antigens. A similar or higher dose of plant-derived HPV-16 L1 VLPs elicited HPV-16 NAb titres of 400-1600 (Maclean *et al.*, 2007; Fernández-San Millán *et al.*, 2008) and plant-derived L1/E6/E7 cVLPS elicited HPV-16 NAb titres of ~400

using a hemagglutination assay (Paz De la Rosa *et al*., 2009). Furthermore, immunisation of humans with the HPV-16 L2 aa 108-120 peptide has shown to elicit HPV-16 NAb titres of 100-1000 (Kawana *et al*., 2003) and mouse antisera from L1/L2 chimaeras containing the L2 epitopes aa 108-120 (Slupetzkey *et al*., 2007) or L2 aa 75-112 and 115-154 (Schellenbacher *et al*., 2009) neutralised homologous HPV-16 PsVs with titres <1000. Therefore the titres obtained in the study are within the range reported by L1/L2 chimaera vaccines produced in other expression systems.

4.4.2.2 Heterologous HPV-18, 45 and 52 neutralisation

Neutralising activity against phylogenically-related HPV-18 and 45 PsV was not detected for all the HPV vaccines (Figure 10-11). Similarly, the L1/L2(56-81) antisera did not neutralise HPV-52 PsV (Figure 12). Although L1/L2(108-120) and L1/L2(17-36) appeared to elicit low HPV-52 NAb titres (50-200), there was a great deal of variation in the assay, possibly due to the purification of partially-assembled PsVs (Figure 7D), and the assay should be repeated to confirm results.

Previous work has demonstrated that L1/L2 chimaeras containing the L2 aa 56-81 peptide cross-neutralises both HPV-18 and 52 (Kondo *et al*., 2008). However, the chimaeras were assembled into cVLPs unlike L1/L2(56-81), suggesting VLP assembly is important to induce the production of high NAb titres. Furthermore, L1/L2 chimaera containing L2 aa 17-36 or 18-36 (Kondo *et al*., 2008; Schellenbacher *et al*., 2009) elicits NAb against HPV-18, 45 and 52. However, the L2 peptides were inserted into the DE loop (Schellenbacher *et al*., 2009) and the dosage was not stated for the study conducted by Kondo et al. (2008). In this study, the low HPV-52 NAb titres elicited by plant-derived L1/L2(17-36) in mice were comparible to titres elicited by a similar L1/L2 chimaera expressed in insect cells (Schellenbacher *et al*., 2009), suggesting the expression system does not affect the ability of the antigen to cross-neutralise HPV-52.

Plant-derived L1/L2(108-120) chimaera appeared to elicit HPV-52 NAb and may have potential as a cross-protective HPV vaccine, supported by evidence that the L2 aa 108-120 peptide has been shown to elicit HPV-52 NAb titres of 50-1000 respectively in humans (Kawana *et al.,* 2003). There is no evidence that HPV-16 L2 aa 108-120 cross-neutralises HPV-45, however L1/L2 chimaeras containing similar L2 aa 96-115 or 75-112 epitopes cross-neutralised phylogenically-related HPV-18 (Kondo *et al*., 2008; Schellenbacher *et al*., 2009). However NAb titres reported in the studies were low (<100) and it is possible that elicited HPV-18 NAb were too low to detect in the L1/L2(108-120) antisera.

4.4.3 Conclusion

Plant-derived HPV-16 L1 VLPs elicit the highest anti-L1 and HPV-16 NAb titres in mice. Although both L1/L2(108-120) and L1/L2(17-36) chimaeras elicit anti-L1 and L2 responses, L1/L2(108-120) assembled into cVLPs and appears to be the best candidate vaccine, as it elicits high anti-L1 responses and was the only chimaera to elicit both HPV-16 NAb and cross-neutralise heterologous HPV-52. L1/L2(56-81) did not appear to be immunogenic and does not show potential for further development as a HPV vaccine.

Chapter 5: Transgenic expression of L1 chimaeras

5.1 Introduction

One of the main strategies employed in the production of plant-derived HPV antigens is transgenic expression. Stable transformation of plants involves the integration of a foreign gene into the nuclear or plastid genome (Fischer and Emans, 2000). Several studies have expressed HPV-16 L1 in transgenic plants (Appendix A), either by nuclear transformation (Biemelt *et al.*, 2003; Varsani *et al.*, 2003b; Liu *et al.*, 2005; Maclean *et al.*, 2007) or transplastomic chloroplast transformation (Fernández-San Millán *et al.*, 2008; Lenzi *et al.*, 2008; Waheed *et al.*, 2011), and the first plant-expressed HPV-16 L1/E6/E7 chimaera has recently been expressed in transgenic tomatoes (Paz De la Rosa *et al*., 2009).

Low expression yields (<1% TSP) have been consistently reported for HPV-16 L1 expressed in nuclear-transformed transgenic plants, possibly due to post-transcriptional gene silencing (PTGS) and adverse position effects (Rybicki, 2009). Similarly, the L1/E6/E7 chimaera also demonstrated low yields of 0.1% TSP in transgenic tomatoes (Paz De la Rosa *et al*., 2009). However, human codon-optimisation of the HPV-16 L1 gene and targeting the protein to tobacco chloroplasts has significantly improved transgenic yields to >1% TSP, with yields of 500 – 650 mg/kg plant tissue reported for the L1 transgenic lines (Maclean *et al*., 2007). This suggests that the nuclear transformation of tobacco still has potential for the high-level expression of HPV-16 L1 and may be viable for commercial exploitation.

HPV-16 L1 expressed in tobacco chloroplasts via transplastomic transformation produced yields of 3000 mg/kg (24% TSP), the highest plant-expressed HPV-16 L1 yields recorded to date (Fernández-San Millán *et al*., 2008). However, other transplastomic HPV-16 L1 expression studies have reported less impressive yields of 1.5% TSP (Lenzi *et al*., 2008; Waheed *et al*., 2011) and there are several important limitations of this system to consider (Rybicki, 2010; Lössl and Waheed, 2011), including the complexity of

transformation and selection, the limited choice of susceptible host plants, the prokaryotic-like post-translational processing and negative pleiotropic effects, which was recently reported for HPV-16 L1 (Waheed *et al*. 2011). As a result, transplastomic expression systems require further development in order to be utilized for commercial vaccine production, and the molecular processes responsible for the high-level accumulation of foreign protein in chloroplasts appear to be similar irrespective of whether the recombinant protein is transported to the chloroplast by a signal sequence or expressed from a plastid transgene (Fernández-San Millán *et al*., 2008).

The transgenic expression of three human codon-optimised HPV-16 L1 chimaeras was investigated by nuclear transformation of tobacco plants. The chimaeras were targeted to the chloroplast and contained either the L2 epitope aa 108-120 (Kawana *et al*., 1999), the mouse-restricted E7 CTL epitope aa 49-57 (Feltkamp *et al*., 2003), or both the L2 and E7 epitopes. The L1/L2(108-120), L1/E7M and L1/L2/E7M chimaeras were specifically chosen to examine the effect of the L2 or E7 sequence replacement on chimaera expression. Furthermore, the E7 chimaeras containing the mouse-restricted E7 CTL epitope (E7M) was chosen as vaccine efficacy is first demonstrated in animal models (Rybicki, 2009).

5.2 Materials and Methods

5.2.1 Plant transformation and regeneration

The plant line used for transformation and transgenic expression of the L1 chimaeras was *Nicotiana tabacum* cv. Petit Havana SR1. Leaf discs were transformed as described by Horsch *et al*. (1985). Sterilized leaf discs (~0.5 cm^2) were incubated for 20 min in the pTRAkc-rbcs1-cTP chimaera *Agrobacterium* GV3101 cultures (OD_{600} = 1.0), grown overnight at 27ºC in induction medium with antibiotics (30 µg/ml kanamycin, 50 µg/ml rifampicin and 50 µg/ml carbenicillin). Leaf discs were placed on co-cultivation medium (4.421 g/L MS nutrients and vitamins, Highveld Biological; 2 mg/L BAP; 30 g/L

sucrose; 10 g/L plant agar, Sigma; pH 5.8 with KOH) for 2 days at 22ºC under constant light. The tranformation negative controls consisted of leaf discs dipped in induction medium, or dipped in the kanamycin-sensitive *Agrobacterium* GV3101 culture.

Leaf discs were transferred to regeneration media containing kanamycin (300 mg/L) and cefotaxime (400 µg/L; Claforan®) and incubated at 26ºC, 16 hrs light / 8 hrs, until small shoots developed (4–6 weeks). Shoots were separated from the original leaf discs and transferred to rooting medium after 4-8 weeks (4.421 g/L MS nutrients and vitamins, Highveld Biological; 1 mg/L NAA; 30 g/L sucrose; 10 g/L plant agar, Sigma; pH 5.8 with KOH) with kanamycin and cefotaxime selection. When roots and shoots were established (4-6 weeks), regenerated plantlets were transplanted into soil and grown to maturity. Flowers were self-pollinated and the seeds were collected.

5.2.2 PCR detection of the L1 gene

Plant genomic DNA (gDNA) was extracted using the Doyle and Doyle (1987) protocol. After 2 weeks post-transplantation into soil, putative transgenic gDNA was extracted from 3 leaf discs per transgenic plant and plant gDNA was screened for the presence of the HPV chimaera gene by PCR using the HPV L2 or E7 epitope-specific primers and the PCR profile described in Chapter 2 (Section 2.2.4.2). PCR products were separated on a 2% TBE agarose gel and detected using ethidium bromide.

5.2.3 Selection and generation of T_1 transgenic lines

Seeds were collected from each of the regenerated plants (R_0 generation), surface-sterilized and germinated on regeneration medium under kanamycin selection (100 mg/L) for 1 week at 22ºC under constant light, in order to test seed viability and verify the presence of kanamycin-resistant chimaera transgene. To determine the % seed germination, 3 plates containing 50-100 seeds were prepared for each transgenic line. The number of germinated seeds was expressed as a percentage of the total seeds per plate

and the values were averaged. Wild-type Petit Havana SR1 seeds (kanamycin-sensitive) were included as a negative control. Seedlings (T_1 generation) were transferred to soil when the shoots and roots were established and allowed to fully mature.

5.2.4 Protein extraction and chimaera quantification

Protein was extracted from R_0 and T_1 transgenic plants 4 weeks post-transplantation into soil (plants ~20 cm in height). The protein extraction, anti-L1 western blot detection and quantification of the total soluble protein (TSP) in each transgenic plant extract was performed as described for the transient-expressed chimaeras in Chapter 2 (Section 2.2.9).

Supernatants were screened for HPV chimaera expression by CamVir1 (1:10000) western blot analysis. The wild-type Petit Havana SR1 plant extract was included as a negative control. A transiently-expressed L1/L2(108-120) sample (previously quantified using Cervarix), was run as a positive control and a standard on each blot. The density of the bands detected on anti-L1 western blots was measured using GeneTools (SYNGENE) and estimated relative to the western blot standard. The relative chimaera yield was calculated by expressing the total chimaera yield (mg/kg plant tissue) as a percentage of the TSP, and the fold decrease in % TSP yield for the T_1 transgenic lines was calculated relative to the higher-expressing R_0 lines.

5.2.5 Electron microscopy analysis

The structural assembly of transiently and transgenically-expressed L1/L2, L1/E7M and L1/L2/E7M chimaeras was analyzed using immunocapture transmission electron microscopy. For transient chimaera expression, pTRAkc-rbcs1-cTP chimaeras co-expressed with NSs and extracted from *N. benthamiana* 5 days post-infiltration (dpi) was analysed (described in detail in Chapter 2, Section 2.2.6). The highest expressing transgenic line for each chimaera was examined for both the R_0 and T_1 generation (4 weeks post-transplantation into soil). Wild-type Petit Havana SR1 crude extract was

included as a negative control. Crude plant extract was immunotrapped with CamVir1 antibody (1:1000) on carbon-coated copper grids, negatively stained with 2% uranyl acetate and viewed on a Zeiss 912 Omega Cryo EFTEM.

5.3 Results

5.3.1 PCR detection of the L1 gene in R_0 transformants

Several plant lines were regenerated from the transformation of leaf discs with the HPV chimaeras, mediated by *A. tumefaciens*. A few plant lines showed abnormal growth and phenotypical characteristics and were not transplanted into soil. Regeneration of 5-7 primary transformants (R_0) was obtained for each HPV chimaera and regenerated plants displayed normal phenotypical characteristics. The negative control leaf discs on kanamycin-selective regeneration plates show no growth. Transformant gDNA was screened for the presence of the HPV chimaera gene by PCR, using L2 or E7 epitope-specific primers (Figure 1).

Band detection using the plant gDNA was difficult, even after several attempts to optimize the reaction using different concentration of gDNA template, $MgCl_2$ concentrations and DMSO. A commercial “Extract ‘n Amp” gDNA extraction kit was also utilized with no additional success (data not shown). Faint PCR bands were detected for four transgenic lines for the L1/L2, L1/E7M and L1/L2/E7M chimaeras, thus confirming the presence of the transgene. The lines where bands are indistinct or absent are indicated with a star (Figure 1).

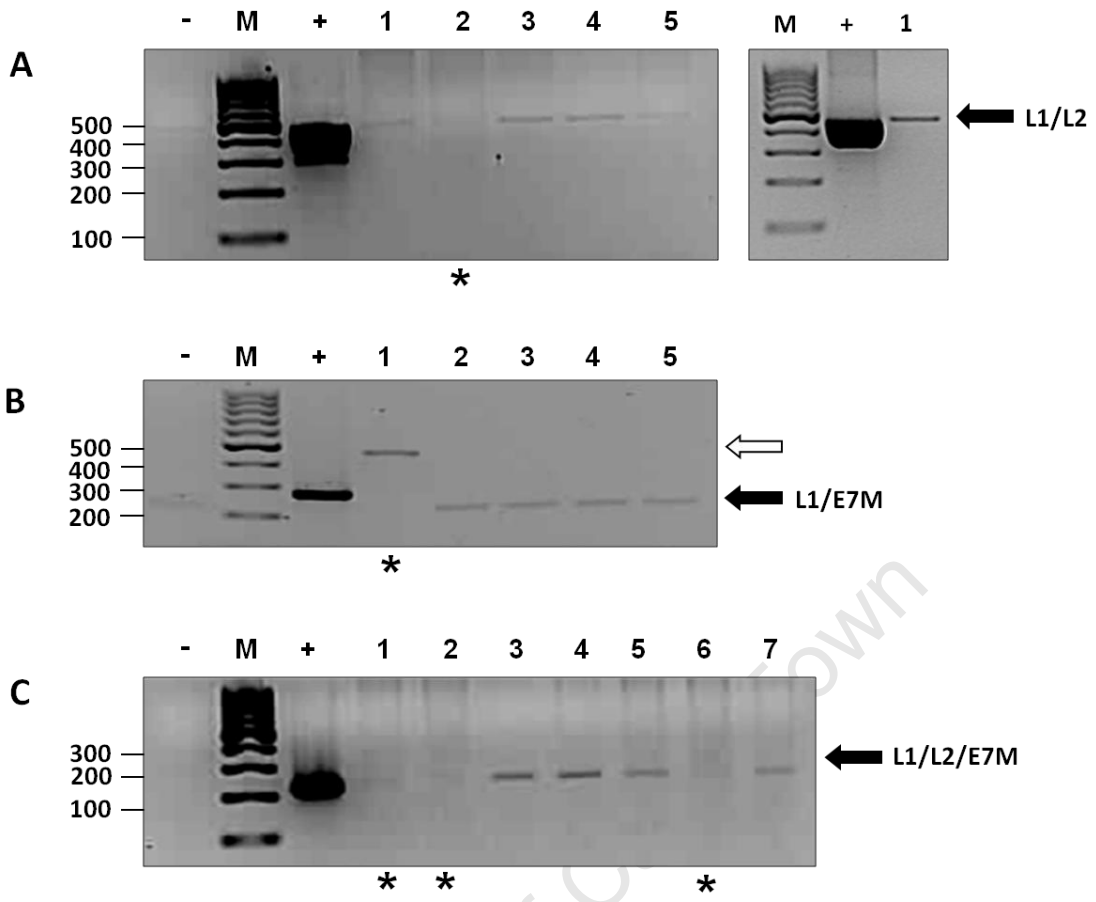

Figure 1: PCR detection of the L1 gene in the R_0 putative transgenic lines for the A) L1/L2, B) L1/E7M and C) L1/L2/E7M chimareas. Labels: M = DNA marker with size in bp indicated on the left. –ve control = no template water control with the appropriate primer set. +ve control = pTRAkc-rbcs-cTP chimarea construct DNA. Numbers 1-7 indicate the putative transgenic lines tested. Black arrows represent the L2 or E7 epitope-specific chimaera PCR products. The white arrow represents a non-specific PCR product. Transgenic lines without a distinct chimaera-specific PCR band are indicated with a star (*)

Southern blotting is required to determine gene copy number and to verify that the transgenic lines are not genetic clones (Warzecha *et al*., 2003; Paz De la Rosa *et al*., 2009). Furthermore, RNA detection using northern blotting (Biemelt *et al*., 2003; Warzecha *et al*., 2003; Paz De la Rosa *et al*., 2009) or RT-PCR (Varsani *et al*., 2003b; Kohl *et al*., 2007) would also be useful to detect transcription of the chimaera transgene. However, this was not done in this study due to time constraints.

5.3.2 Production of the T_1 transgenic lines

5.3.2.1 T_1 seed germination

Seeds from each transgenic line were germinated under kanamycin selection, in order to test seed viability and verify the presence of kanamycin-resistant chimaera transgene. Wild-type Petit Havana SR1 seeds (kanamycin-sensitive) were included as a negative control. Although the wild-type seeds initially germinated, the seedlings died after 6 weeks, unlike the putative transgenic T_1 seedings. The comparison is shown in Figure 2, using a transgenic L1/L2 line.

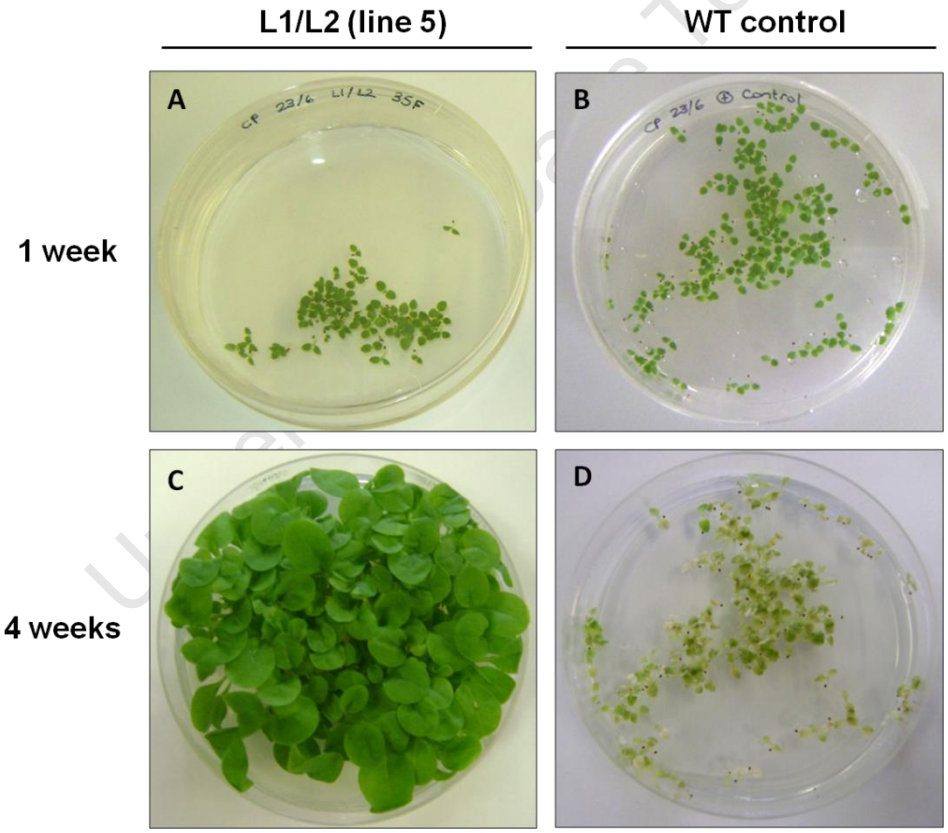

Figure 2: T_1 seed germination of a transgenic L1/L2 line (kanamycin-resistant) in comparison to the wild-type (WT) Petit Havana SR1 negative control (kanamycin-sensitive) under kanamycin selection. Both seeds germinated at 1 week (2A-B). However, the WT seedlings did not continue to grow, as seen at 4 weeks (2C-D), and died after 6 weeks.

All HPV chimaera transgenic lines germinated (an example of the seedling regeneration plates for each of the HPV chimaeras is shown in Figure 3A-C), and were transplanted into soil (Figure 3D).

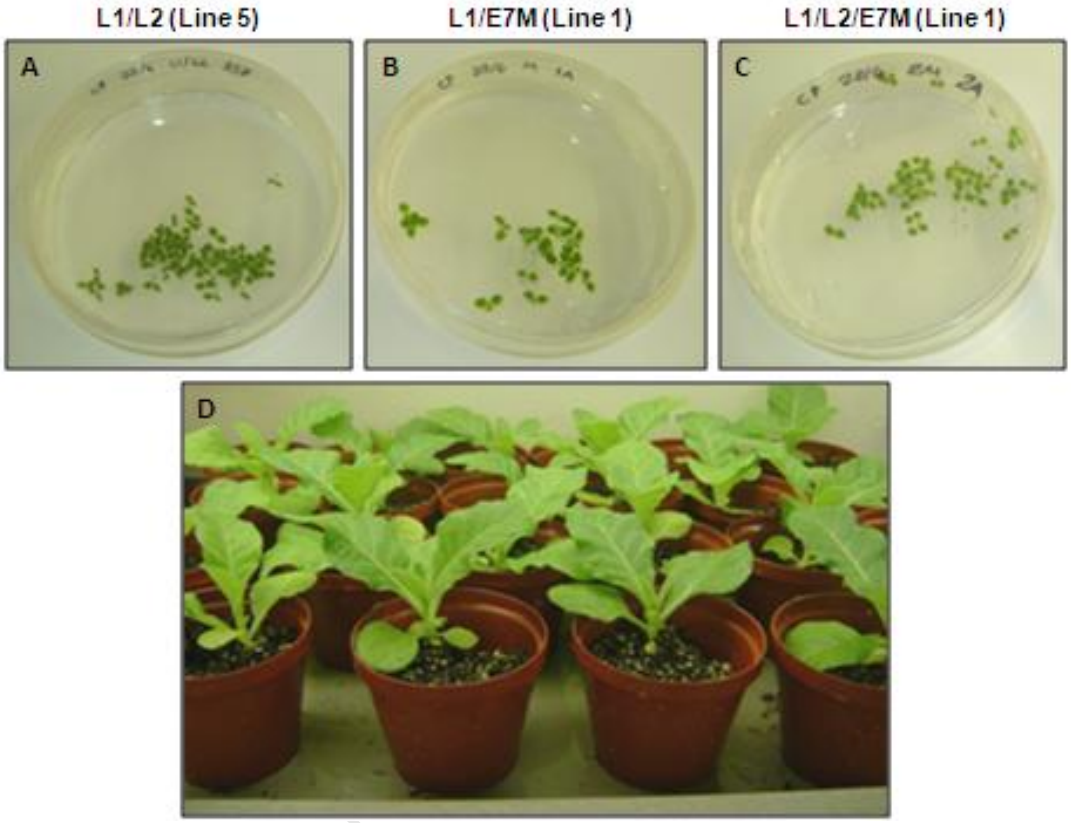

Figure 3: T_1 seed germination of transgenic chimaeras under kanamycin selection. Each chimaera line was tested and examples of germinating transgenic A) L1/L2, B) L1/E7M and C) L1/L2/E7M chimaera seeds are shown, with D) the fully developed T_1 plants. The wild-type Petit Havana SR1 seeds (negative control) initially germinated but did not survive under kanamycin selection.

5.3.2.2 R_0 and T_1 HPV chimaera expression and quantification

Protein was extracted from R_0 and T_1 transgenic plants and supernatants were screened for L1 chimaera expression by CamVir1 (1:10000) western blot analysis, using the transiently-expressed L1 chimaeras as positive expression controls (Figure 4A-C). The wild-type Petit Havana SR1 plant extract, included as a negative control, was not detected (Figure 4).

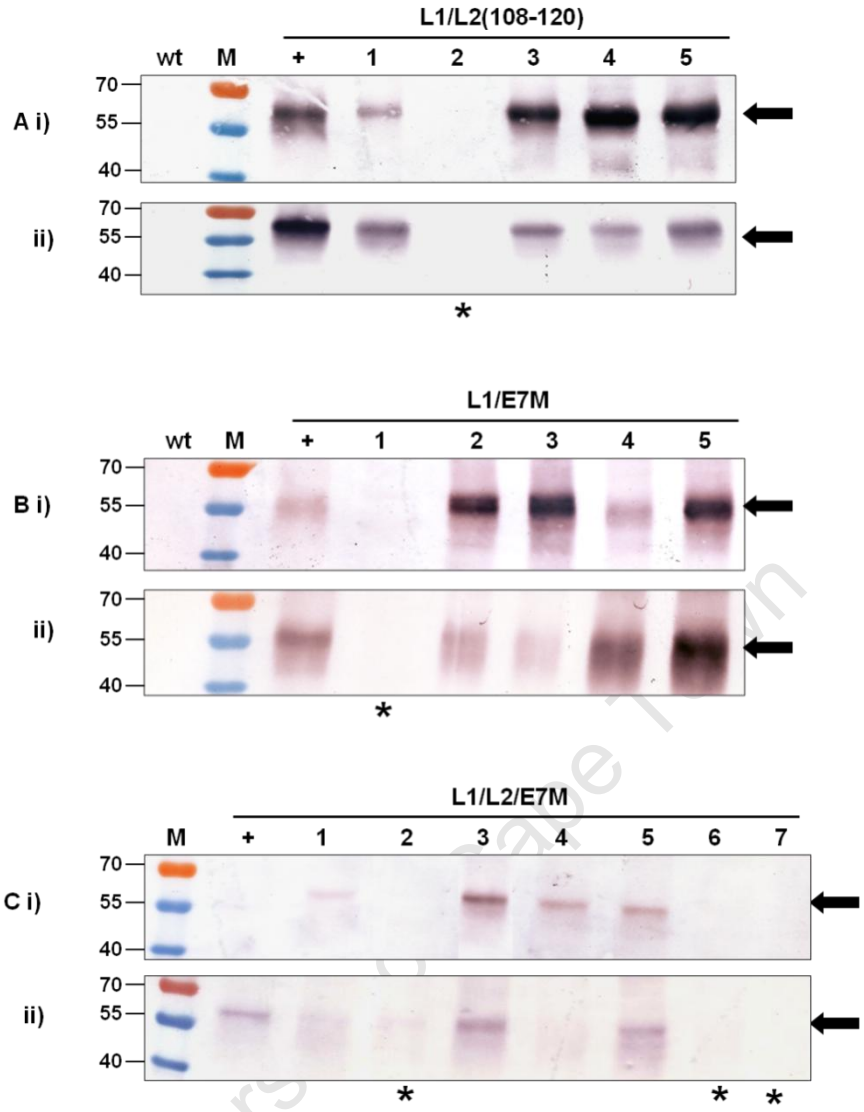

Figure 4: Expression of A) L1/L2, B) L1/E7M and C) L1/L2/E7M chimaera R_0 and T_1 putative transgenic lines. Chimaera expression was detected by anti-L1 western blot analysis for both the i) R_0 and ii) T_1 crude plant extracts. Labels: M = protein marker with size in kDa indicated on the left. –ve control = wild-type Petit Havana SR1 crude plant extract. +ve control = chimaeras transiently-expressed using pTRAkc-rbcs-cTP and co-infiltrated with NSs (5 dpi). Numbers 1-7 indicate the chimaera putative transgenic lines tested. Black arrows represent the detected chimaera (~56 kDa). Transgenic lines without a distinct HPV chimaera band are indicated with a star (*).

A quantified L1/L2(108-120) chimaera standard was included in all the western blots (data not shown), in order to estimate chimaera expression yields and determine the relative chimaera yield (expressed in mg/kg plant tissue and % TSP) for each transgenic line (Table 1). The fold decrease in % TSP yield for the T_1 transgenic lines was calculated relative to the higher-expressing R_0 lines.

Table 1: Summary of the HPV chimaera putative transgenic lines

				Regeneration (R_0)		First generation (T_1)	
HPV chimaera	Putative lines	PCR* detection	WB** detection	Yield (mg/kg)	% TSP***	% seed germination	Fold decrease in % TSP**
L1/L2	1	Y	Y	110	1.2	71	1.1
	2	N	N	-	-	69	-
	3	Y	Y	590	4.4	78	4.4
	4	Y	Y	730	7.4	64	12.3
	5	Y	Y	760	6.4	76	5.3
	Summary	4/5 lines	4/5 lines	110 – 760	1.2 - 7.4	60 – 80	1 – 12
L1/E7M	1	N	N	-	-	91	-
	2	Y	Y	180	1.7	89	17.0
	3	Y	Y	220	2.3	79	4.6
	4	Y	Y	50	0.5	85	1.3
	5	Y	Y	210	1.8	78	3.0
	Summary	4/5 lines	4/5 lines	50 – 220	1.7 - 2.3	80 – 90	1 – 17
L1/L2/E7M	1	N	Y	15	0.06	83	-
	2	N	N	10	0.02	89	-
	3	Y	Y	40	0.10	84	9.0
	4	Y	Y	10	0.02	91	30.0
	5	Y	Y	30	0.10	67	6.0
	6	N	N	5	0.01	68	-
	7	Y	N	10	0.01	90	-
	Summary	4/7 lines	4/7 lines	5 – 40	0.01 - 0.10	70 – 90	6 – 30

*Detection of the HPV chimaera genes by PCR (Y = yes, N = no).
**Detection of the HPV chimaera protein by western blotting (WB).
***TSP = Total soluble protein

PCR detection of the chimaera transgenes identified four putative transgenic lines for each of the L1 chimaeras (Figure 1, Table 1). Western blot detection, using the anti-L1 MAb CamVir1, confirmed these transgenic lines for both the L1/L2 chimaera (Figure 4A: line 1 and 3-5) and L1/E7 chimaera (Figure 4B: line 2-5).

The results for L1/L2/E7M were conflicted. Only 3 putative transgenic lines appeared to contain both the L1/L2/E7M gene and the expressed protein (Figure 4C: line 3-5). However, expression in line 4 was not present in the T_1 generation, indicating that the gene may have been lost or experienced gene silencing. Line 7 appeared to contain the L1/L2/E7M gene (Figure 1C)

but expressed protein was not detected (Figure 4C), suggesting the transgene may have also been silenced. In contrast, line 1 was not detected by PCR (Figure 1C), although very low levels of protein were expressed (Figure 4C). It is possible the L1/L2/E7M transgene is present in line 1 and was not detected by PCR, suggesting further work is needed to optimize the PCR experiments and confirm the presence of the transgene.

As described for transient expression (Chapter 2), the L1/L2 chimaera was generally expressed to higher levels than the E7M chimaeras, with higher yields obtained for the L1/E7M than L1/L2/E7M (Figure 4A-C, Table 1). The maximum L1/L2 yield was 4-fold higher than the maximum L1/E7M yield, suggesting the sequence and length of the sequence insertion affects expression. L1/L2 was up to 19-fold higher than the highest L1/L2/E7M yield. This suggests that the insertion of 2 epitopes within the L1 structure appears to negatively impact protein expression. All transgenic chimaeras showed higher expression levels in the R_0 generation, with a decrease in the % TSP yield in the T_1 generation (Table 1).

The L1/L2 R_0 transgenic lines produced yields of 110 – 760 mg/kg plant tissue (Table 1). Although the T_1 generation shows a up to a 12-fold reduction in yield, transgenic lines 1, 4 and 5 still have yields >1% TSP (data not shown), considered the threshold for the commercial production of antigens (Fischer *et al*., 2004). The L1/E7M R_0 transgenic lines demonstrated lower yields, with only line 2 and 3 containing yields >1% TSP (Table 1). However, yields were <1% TSP in the L1/E7M T_1 generation (0.1 – 0.6 %TSP; data not shown) and in all the L1/L2/E7M transgenic lines (<0.1% TSP), suggesting the L1/E7M and L1/L2/E7M transgenic plants are not suitable for commercial exploitation.

All T_1 putative transgenic seeds germinated (64-91% germination), even for the lines which don't appear to contain the transgene (L1/L2 line 2, L1/E7M line 1, L1/L2/E7M line 2 and 6). The reason for this may be because the kanamycin concentration was too low to allow for efficient transgene selection, as suggested by the initial germination and growth of the kanamycin-sensitive

wild-type Petit Havana SR1 seeds (negative control), and thus higher kanamycin concentrations need to be utilized in the future.

5.3.3 Electron microscopy analysis of L1 chimaera assembly

Immunocapture electron microscopy was used to analyze the structural assembly of the L1 chimaeras targeted to the tobacco chloroplasts (Figure 5). The highest-expressing transgenic line was examined for each of the L1 chimaeras and transiently-expressed L1 chimaeras were included for comparative purposes. Wild-type Petit Havana SR1 crude plant extract was included as a negative control.

Figure 5A shows the L1/L2 chimaera assembled into cVLPs. Transiently-expressed L1/L2 assembled into distinctive small cVLPs and capsomere aggregates, as observed in Chapter 3 (Section 3.3.3). The transgenic expression of L1/L2 resulted in chimaeras assembling into more distinctive cVLPs (~50 nm) and a variety of secondary structures, including non-uniform capsomere aggregates and partially-formed cVLPs.

The transgenic L1/E7M chimaeras assembled into distinct, regular-sized cVLPs. In contrast, the majority of transiently-expressed L1/E7M chimaeras assembled into capsomeres (Figure 5B), although a few cVLPs were observed in the crude plant extract (inset in Figure 5B).

The low-expressing L1/L2/E7M chimaeras (Figure 5C) assembled into distinctive cVLPs when expressed either transgenically or transiently, although the majority of the chimaeras formed capsomeres and aggregates, particularly for the transiently-expressed chimaera.

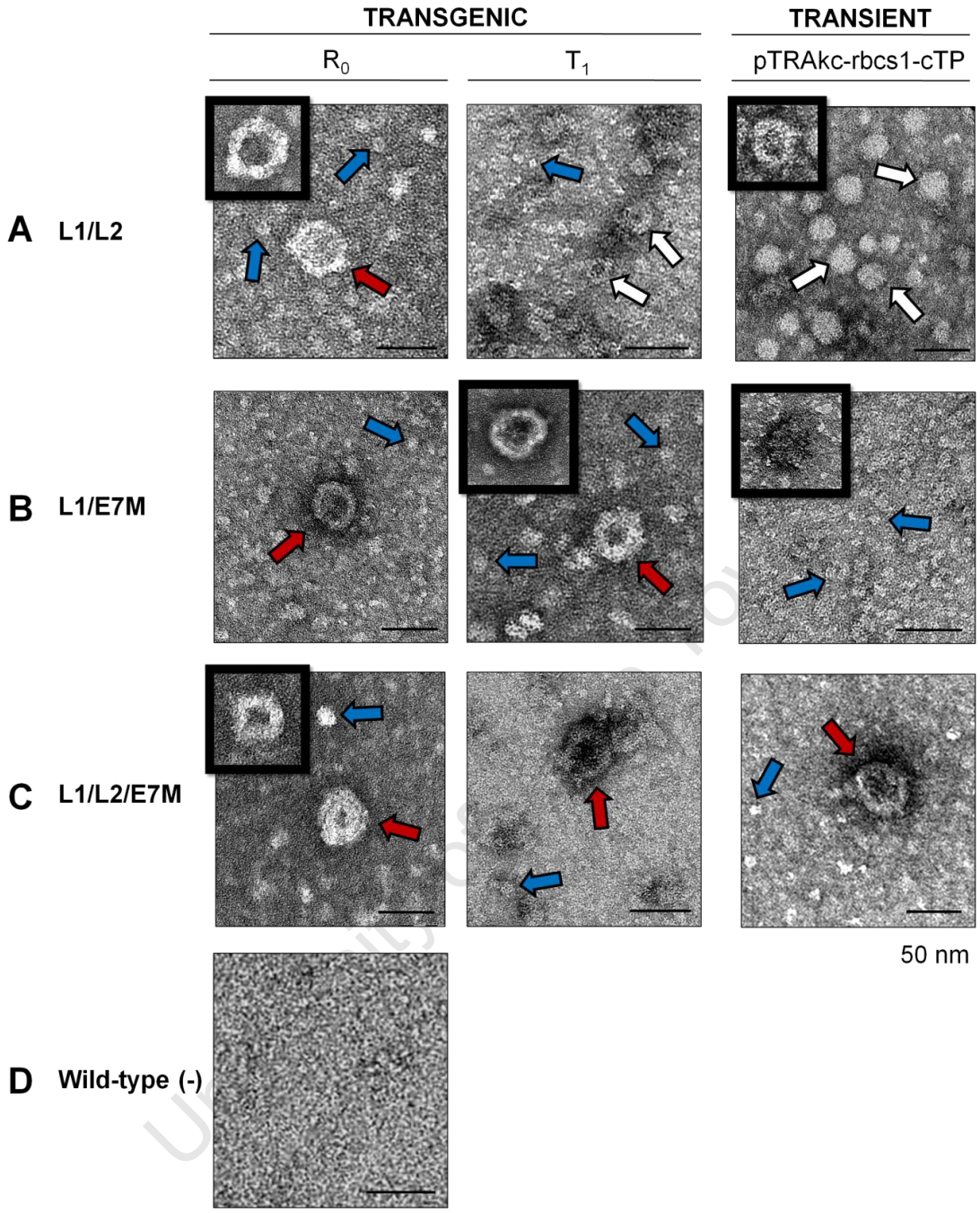

Figure 5: Transmission electron micrographs of immunotrapped A) L1/L2, B) L1/E7M and C) L1/L2/E7M chimaeras, either transiently-expressed in the presence of NSs and extracted 5 dpi, or expressed transgenically in tobacco plants. For the transgenic chimaeras: the transgenic line with the highest expression yields (L1/L2 line 5, L1/E7M line 5 and L1/L2/E7M line 2) was examined for both the R_0 and T_1 generations. D) Wild-type Petit Havana SR1 crude plant extract served as the negative control. The insets emphasize the distinctive chimaeric VLPs observed in the samples. Red arrows indicate full-size VLPs (50-55 nm). Blue arrows indicate capsomeres (~10 nm) and white arrows indicate capsomere aggregates or small VLPs (20-45 nm). Scale bar = 50 nm. The chimaeras were analyzed on a Zeiss 912 Omega Cryo EFTEM.

5.4 Discussion

Transgenic expression of L1 or L1-based chimaera candidate vaccines is a potential strategy for the cost-effective production of HPV vaccines (Biemelt *et al.*, 2003; Varsani *et al.*, 2003b; Liu *et al.*, 2005; Paz De la Rosa *et al.*, 2009) and three L1 chimaeras (L1/L2, L1/E7M and L1/L2/E7M) were expressed in transgenic tobacco plants and analysed in this study.

5.4.1 Production and genetic analysis of the putative R_0 transgenic lines

PCR detection of the chimaera genes identified four putative transgenic lines for each chimaera (Figure 1). Several problems were experienced with PCR, despite using similar primers and reaction conditions for the detection of *E. coli* and *A. tumefaciens*-expressed L1 chimaera genes (Chapter 2, Section 2.2.4.2). Optimisation experiments using a range of template DNA, $MgCl_2$, DMSO concentrations were largely unsuccessful, suggesting the plant gDNA may contain extract contaminants that inhibit PCR. However, another commercial extraction method was similarly unsuccessful and further work is warranted. Time constraints prevented further PCR optimisation, as well as Southern blotting and RNA analysis.

5.4.2 Production of L1 chimaera T_1 transgenic plants

To assess the inheritance of the trangene in the T_1 generation, 5-7 plant lines for each HPV chimaera were self-pollinated and the seeds were screened by kanamycin-resistance to select the lines containing the chimaera transgenes. All lines germinated (Table 1), including the kanamycin-sensitive negative control (wild-type Petit Havana SR1 seeds), which suggests the kanamycin concentration may not have been sufficiently high to select for antibiotic resistance. However, the negative control seedlings did not continue to develop after initial germination (Figure 2) and died within 6 weeks using the kanamycin-supplemented media, unlike the putative transgenic T_1 seedings.

5.4.3 Transgenic expression of the L1 chimaeras

Four L1/L2 and L1/E7M transgenic lines expressed the chimaeras at detectable levels (Figure 4: L1/L2 line 1 & 3-5, L1/E7M lines 2-5). This was

observed for both the R_0 and T_1 generation (Figure 4, Table 1), suggesting the L1/L2 and L1/E7M transgene is stably integrated in the T_1 generation. There appears to be at least four L1/L2/E7M transgenic lines (Table 1: Line 3-5 &7), although chimaera expression was not detected for line 7, and in the T_1 generation for line 4 (Figure 4C), possibly due to position effects, gene silencing or even gene instability. As a result, it appears that the L1/L2/E7M chimaera transgene is less stable in tobacco plants.

The transgenic expression of the L1 chimaeras was more successful than the plant-derived L1/E6/E7 chimaera recently expressed in transgenic tomatoes (Paz De la Rosa *et al*., 2009). All the L1/L2 and L1/E7 transgenic lines and two of the L1/L2/E7M lines (line 3 & 5) gave yields similar or greater than 0.1% TSP (Table 1), the highest yield for a plant-derived L1-based chimaera.

The L1/L2 chimaera was expressed to higher levels than L1/E7M in both transient (Chapter 2) and transgenic expression systems (Figure 4, Table 1). Transient expression of L1/L2(108-120) and L1/E7M gave yields of ~1000 mg/kg and 30 mg/kg plant tissue respectively (Chapter 2, Figure 12 and Table 4), and expression of L1/L2 in 3 of the 4 transgenic lines demonstrated higher expression yields than the highest-expressing L1/E7M line (L1/L2 Line 3-5: 590-760 mg/kg, vs. L1/E7M Line 3: 220 mg/kg). This suggests the sequence and the length of the epitope insertion may affect the expression of L1-based chimaeras when epitopes are located in similar L1 positions. Furthermore, the maximum L1/L2 transgenic yields (Table 1: 760 mg/kg) are similar to the HPV-16 L1 yields (500 - 650 mg/kg) expressed in transgenic tobacco plants by Maclean *et al*. (2007), suggesting the L2 epitope may have a smaller affect on expression and protein stability in comparison to the E7 epitope.

The L1/L2/E7M chimaera, containing 2 epitope insertions, was poorly expressed in both transient and transgenic systems, with yields of 5-40 mg/kg plant tissue (Chapter 2, Table 4; Chapter 5, Table 1). In comparison, the

L1/L2 chimaera produced yields up to 19-fold higher than L1/L2/E7M (Table 1), suggesting that the second epitope insertion is not well-tolerated.

5.4.4 Structural assembly of the L1 chimaeras

Transmission electron microscopy was used to analyze the structural assembly of transiently and transgenically-expressed L1/L2, L1/E7M and L1/L2/E7M chimaeras (Figure 5). Wild-type Petit Havana SR1 plant extract was included as a negative control and no capsomere-like or VLP-like structures were observed in this sample.

All the L1 chimaeras assembled into higher-order structures (Figure 5), with transgenic plant-derived L1/L2, L1/E7M and L1/L2/E7M assembling into cVLPs (~50 nm), similar to the HPV-16 L1 VLPs derived from transgenic plants (Biemelt *et al.*, 2003; Varsani *et al.*, 2003b). Transiently-expressed L1 chimaeras assembled into capsomere aggregates and cVLPs of various sizes (25-50 nm). As a result, sequence replacements of ≤13 residues into the h4 region and 9 residues into the coil between the h4 and β-J structural region do not appear to prevent capsomere and VLP assembly in transgenic plants, although the cVLPs are not morphologically similar to native virions.

5.4.5 Conclusions

Four putative tobacco transgenic lines were obtained for each of the HPV-16 L1/L2, L1/E7M and L1/L2/E7M chimaeras, although one L1/L2/E7M line did not express detectable levels of protein, possibly as a result of position effects or PTGS. Chimaera expression was reduced in the T_1 generation, suggesting that the transgenes were silenced by PTGS.

The L1/L2 chimaeras gave higher expression levels than L1/E7M chimaeras and only low expression was detected for L1/L2/E7M. This suggests the length and sequence of the epitope insertion affects protein expression and a second 9 residue epitope insertion in the h4 and β-J structural region has a negative impact on expression, a factor which should be considered in the further design of HPV chimaera candidate vaccines.

The L1/L2 and L1/E7M transgenes appear to be stably inherited in the T_1 generation, while the L1/L2/E7M transgene appears to be less stable and may have been lost in one of the transgenic lines. All L1 chimaeras assembled into cVLPs of various sizes, although the morphology was irregular. Only the L1/L2 chimaera produced yields >1% TSP in the T_1 generation (Table 1: Line 1, 4 & 5), considered the threshold for the economic production of antigens (Fischer *et al*., 2004). Further analysis of the L1/L2 chimaera lines is needed to determine long-term transgene stability and PTGS effects, as well as the immunogenicity and safety of the plant-derived candidate vaccine. However, the L1/E7M and L1/L2/E7M transgenic plants are not currently viable for commercial exploitation and the use of alternative expression systems may be required in the future.

Chapter 6: Conclusions

Cervical cancer is a global concern and is the third most common cancer among women worldwide, with the majority of cases occuring in developing countries (Ferlay *et al*., 2010). Southern Africa is considered a high-risk region (Parkin and Bray, 2006) and cervical cancer is the most prevalent cancer in black South African women (Mqoqi *et al*., 2004). Therefore HPV vaccine development is a priority in preventative cervical cancer research, both globally and locally.

There is an incentive to develop cheaper second-generation HPV vaccines which are broadly protective against multiple oncogenic types and therapeutic to treat pre-existing HPV infections and associated disease. This study examined the expression of eight HPV-16 L1-based chimaeras containing cross-neutralising L2 epitopes and therapeutic E7 CTL epitopes in plants and analyzed the immunogenicity of three plant-derived L1/L2 chimaera candidate vaccines in mice.

Three types of HPV-16 L1 chimaeras were analyzed: the L1/L2, L1/E7 and L1/L2/E7 chimaeras. The L1/L2 chimaeras consisted of the South African HPV-16 L1 sequence containing cross-neutralising L2 epitopes; either HPV-16 L2 aa 108-120, 56-81 or 17-36, or the BPV-1 L2 aa 1-88 epitope. The L1/E7 chimaeras contained HPV-16 E7 CTL epitopes comprising of either the mouse-restricted E7 aa 49-57 epitope (E7M) or human-restricted E7 aa 86-93 epitope (E7H). The L2 or E7 epitopes in the L1/L2 and L1/E7 chimaeras replaced the h4 region of L1 at aa 414, with the exception of the L1/L2 chimaera containing the BPV-1 L2 epitope which replaced the entire L1 C-terminal. The L1/L2/E7 chimaeras contained L2 aa 108-120 epitope in the same h4 region and the E7 epitope in the coil between the h4 helix and ß-J structural region at aa 433/434. These two insertion positions were chosen as they have been shown to successfully display L2 epitopes and elicit both anti-L1 and L2 humoral immune responses in mice (Varsani *et al.*, 2003a).

6.1 Expression of L1 chimaeras in plants

Plant expression systems present a cost-effective alternative for the production of vaccine antigens. However, optimisation of protein expression is necessary to obtain yields >1% TSP, considered the threshold for commercial production of recombinant proteins in plants (Fischer *et al*., 2004).

6.1.1 Transient expression

The highest HPV-16 L1 yields obtained thus far in an *Agrobacterium*-mediated transient expression system was 530-550 mg/kg plant tissue. These yields were acheived by human codon-optimisation of the L1 gene and by two strategies: targeting the expressed protein to the chloroplast (Maclean *et al*., 2007) or using an agroinfiltration-delivered self-replicative BeYDV vector (Regnard *et al*., 2010). This is the first study which directly compared these strategies using eight human codon-optimised L1 chimaeras.

All the HPV L1/L2, L1/E7 and L1/L2/E7 chimaeras were expressed in *N. bethamiana* using an *Agrobacterium*-mediated transient system. Optimisation experiments indicate that co-expression of the NSs silencing suppressor increased expression levels and the use of a BeYDV-based self-replicative vector increases the accumulation of L1/L2 chimaeras in the cytoplasm in comparison to a non-replicative vector. However, targeting the expressed chimaeras to the chloroplast is the best strategy for the high-level accumulation of chimaeras in plants and improved yields by up to 28-fold.

The L1/L2 chimaeras, containing the HPV-16 L2 epitope sequences aa 108-120, 56-81 or 17-36, were highly-expressed and produced commercially viable yields of ~1200 mg/kg (>1% TSP) which were 2-fold higher than the maximum HPV-16 L1 yields reported in a similar plant expression study (Maclean *et al*., 2007). However, the L1/L2 chimaera with the BPV L2 aa 1-88 epitope was not well-expressed and degradation was detected, suggesting expression of this chimaera is not viable in plants.

The low-expressing L1/E7 and L1/L2/E7 chimaeras produced maximum yields of 30 – 80 mg/kg plant tissue (<1% TSP) and thus require further optimisation in this expression system.

6.1.2 Transgenic expression

Transgenic expression of HPV-16 L1 or L1-based chimaeras is a strategy for the production of HPV vaccine antigens. Although nuclear expression of HPV-16 L1 is traditionally associated with low yields (<1% TSP), human codon-optimisation of the HPV-16 L1 gene and targeting the protein to tobacco chloroplasts significantly improved yields to 500 - 650 mg/kg plant tissue in L1 transgenic lines (>1% TSP, Maclean *et al*., 2007), suggesting nuclear transgenic expression of HPV antigens may still be viable for commercial exploitation.

Three types of L1-based chimaeras were transgenically-expressed in nuclear-transformed tobacco plants: L1/L2(108-120), L1/E7M and L1/L2/E7M. The chimaeras were all targeted to the chloroplasts and were specifically chosen to examine the effect of the L2 or E7 sequence replacement on chimaera expression.

Four transgenic lines were obtained for each of the HPV chimaeras. Similar to transient expression, L1/L2(108-120) was expressed to higher levels than L1/E7M and low expression was detected for the L1/L2/E7M, with maximum yields of 760, 220 and 40 mg/kg plant tissue respectively. Chimaera expression was reduced in the T_1 generation, possibly as a result of PTGS, and only the L1/L2(108-120) chimaera lines continued to produce commercially viable yields >1% TSP.

6.1.3 The effect of epitope insertions on expression

The replacement of the L1 C-terminal with the BPV-1 L2 aa 1-88 epitope resulted in low expression levels and degradation in the plant cytoplasm, suggesting that the replacement of a large portion of the HPV-16 L1 protein (88 residues) affects the expression and/or stability of the chimaera in plants.

The C-terminal plays a role in the assembly of capsomeres and VLPS (Zhou *et al*., 1991b; Varsani *et al*. 2006b; Bishop *et al*. 2007a), thus it is likely that the protein did not assemble and was degraded by cellular proteases (Chen *et al*., 2000). As a result, the production of L1/L2 BPV(1-88) chimaera production in plants was not pursued further.

In contrast, the L1/L2 chimaeras containing the HPV-16 L2 epitopes aa 108-120, 56-81 and 17-36 (13-26 residues) were highly-expressed and produced yields up to 20-fold higher than the other chimaeras in a transient expression system. HPV-16 L1 demonstrated higher yields than the L1/L2 chimaeras, although the differences were not statistically significant. This suggests the L2 epitope substitutions do not affect the expression and accumulation of HPV protein in chloroplasts.

The L1/E7 and L1/L2 chimaeras, containing different epitopes inserted into similar L1 regions, demonstrated different expression levels. This provides further evidence that the expression of recombinant proteins in plants is empirical (Rybicki, 2010). In addition, both transient and transgenic expression of the L1/L2/E7 chimaeras provide evidence that the insertion of a second epitope into the coil between h4 and the β-J structural region in HPV-16 L1 appears to have a negative impact on expression and decreases yields by up to 19-fold in comparison to L1/L2(108-120), a factor which should be considered in the further design of HPV chimaera candidate vaccines. Furthermore, the inheritance of the L1/L2/E7M transgene appears to be less stable in transgenic plants and L1/L2/E7M demonstrated the greatest decrease in expression between the R_0 and T_1 transgenic lines.

6.2 Structural assembly of the L1/L2 chimaeras

The three highly-expressed L1/L2 chimaeras containing the HPV-16 L2 epitopes aa 108-120, 56-81 and 17-36 were chosen as vaccine antigens for the mouse immunogenicity studies and the assembly of the L1/L2 chimaera antigens was examined to determine the presence of higher-order structures.

Plant-derived HPV-16 L1 and the L1/L2 chimaeras were bound by the conformational-specific MAb H16.V5 (Christensen *et al*., 1996), suggesting the chimaeras assembled into higher-order structures (Carter *et al*., 2003; Wang *et al*. 2003; Ryding *et al*., 2007). Electron microscopy confirmed chimaera assembly; however, it was apparent that the L1/L2 chimaeras assembled into a variety of heterologous structures such as capsomeres, aggregates and VLPs. As a result, size-based purification methods were inefficient and HPV-16 L1 and L1/L2 chimaeras were purified using heparin chromatography.

The L1/L2 chimaeras contained L2 epitopes which varied in length. L1/L2(108-120, L1/L2(56-81) and L1/L2(17-36) contained L2 epitope sequence replacements of 13, 26 and 20 residues respectively. Unmodified plant-expressed HPV-16 L1 assembled into T=7 VLPs (~50 nm), which were similar in size and morphology to those expressed in other studies (Biemelt *et al.*, 2003). The L1/L2(108-120) chimaera assembled into smaller cVLPs which varied in size (25-40 nm) while the L1/L2(56-81) chimaera, with the largest sequence replacement, assembled exclusively into capsomeres (~10 nm). The majority of L1/L2(17-36) chimaeras assembled into capsomeres and aggregates, although a low concentration of amorphous cVLPs were observed.

These results suggest the length of the L2 epitope affects the assembly of chimaeras, with epitopes >13 residues having a significant impact on VLP assembly. This is further supported by the electron microscopy results for plant-derived L1/E7M. The chimaera contained a 9 residue E7 epitope and also assembled into VLPs when expressed in transient or transgenic plant systems.

This phenomenon is explained by the presence of a disulphide bond between the highly conserved cysteine residue 175 and 428 in VLPs (Li *et al*., 1998; Sapp *et al*., 1998; Fligge *et al*., 2001). Mutations of these cysteines results in

the formation of capsomeres rather than VLPs (Li *et al*., 1998; McCarthy *et al*., 1998; Sapp *et al*., 1998; Fligge *et al*., 2001; Varsani *et al*., 2006b) and thus the L1/L2(108-120) and L1/E7M chimaeras, with the epitope located at aa 414-426 and aa 417-425 respectively, does not replace the cysteine at residue 428 and does not affect assembly into cVLPs. As a result, the length of the epitope in the h4 region of L1 is an important factor to consider in the future design of L1/L2 chimaera vaccines.

6.3 Immunogenicity of the L1/L2 chimaeras

Plant-derived HPV-16 L1 and L1-based chimaeras have been shown to assemble into immunogenic higher-order structures and elicit the production of NAb (Maclean *et al.*, 2007; Fernández-San Millán *et al.*, 2008; Paz De la Rosa *et al*., 2009), widely considered the gold standard for demonstrating the potential of prophylactic HPV candidate vaccines (Rybicki, 2010) as protection is predominantly mediated by NAb directed against conformational epitopes (Breitburd *et al*., 1995; Jansen *et al*., 1995; Suzich *et al*., 1995; Christensen *et al*., 1996; Kirnbauer *et al*., 1996). Furthermore, insect cell-produced L1/L2 chimaeras with the L2 epitope substituted into the h4 region of L1 were immunogenic and elicited the production of anti-L1 and anti-L2 humoral responses (Varsani *et al*., 2003a). As a result, the immunogenicity of plant-derived HPV-16 L1 and three L1/L2 chimaeras was investigated in this study.

Plant-derived HPV-16 L1 elicited the highest anti-L1 titres, followed by L1/L2(108-120) and L1/L2(17-36). In addition, both L1 and L1/L2(108-120) elicited NAb against homologous HPV-16, which is detected in 61% of invasive cervical cancers worldwide (de Sanjosé *et al*., 2010). L1/L2(108-120) and L1/L2(17-36) demonstrated anti-L2 responses, which suggests the L2 peptides were effectively displayed on the surface of the chimaeric protein. Although none of the antisera cross-neutralised HPV-18 or 45 pseudovirions, both L1/L2(108-120) and L1/L2(17-36) elicited cross-neutralising antibodies against HPV-52, as described by other studies (Kawana *et al.,* 2003; Kondo *et*

al., 2008; Schellenbacher *et al*., 2009). This result is particularly significant in a local context, as HPV-52 is highly prevalent in low and high-grade cervical lesions in South African women (Allan *et al*., 2008).

As a whole, L1/L2(108-120) appears to be the best candidate vaccine as it elicited high anti-L1 titres, elicited an anti-L2 response and was the only chimaera to elicit both HPV-16 NAb and cross-neutralise heterologous HPV-52, unlike the type-specific L1 vaccine. L1/L2(56-81) did not appear to be immunogenic and this chimaera does not show potential for further development as a HPV vaccine.

6.4 The effect of chimaera assembly on immunogenicity

There appears to be a correlation between VLP assembly and L1 immunogenicity. This was demonstrated by Thönes *et al*. (2008), where immunisation with VLPs elicits 20 to 40-fold higher humoral responses than capsomere vaccines. Similarly, plant-derived L1 VLPs elicited the highest anti-L1 response and HPV-16 NAb titres in comparison to the L1/L2 chimaeras, comprising of smaller cVLPs, aggregates and capsomeres. Furthermore, L1/L2(108-120) cVLPs elicited higher anti-L1 titres than L1/L2(17-36) assembled into a mixed population of capsomeres, aggregates and cVLPs, which in turn elicited higher anti-L1 titres than the L1/L2(56-81) capsomeres.

Only L1/L2(108-120) elicited detectable levels of HPV-16 and 52 NAb. Although the L2 aa 17-36 and L2 aa 56-81 have been shown to neutralise the HPV-16, 18, 45 and 52 types used in the neutralisation assays (Gambhira *et al*., 2007b; Kondo *et al*., 2007, 2008; Alphs *et al*., 2008; Schellenbacher *et al*., 2009; Rubio *et al*., 2009) the partial assembly of the plant-derived L1/L2 chimaeras may have affected L2 immunogenicity. L2 peptides fused to keyhole limpit haemocyanin (KLH) rather than displayed on the surface of HPV-16 L1 cVLPs demonstrated lower HPV-16 and 18 NAb titres (Kondo *et al*., 2007, 2008) and denaturation of L1/L2 chimaeras containing L2

aa 17-36 reduced both homologous HPV-16 and heterologous HPV-18, 45 and 52 NAb titres to such an extent that HPV-45 and 52 NAb were not detected in the rabbit antisera (Schellenbacher *et al*., 2009). As a result, there is strong evidence that assembly of L1/L2 chimaeras into cVLPs elicits stronger immune responses than capsomeres and unassembled L1.

6.5 Conclusions and future work

Eight HPV L1-based chimaeras were transiently expressed in plants using an *Agrobacterium*-mediated transient system. However, the L1/L2 chimaera containing a C-terminal BPV-1 L2 aa 1-88 epitope replacement was unstable in plants and was eliminated as a potential candidate vaccine. The dual prophylactic and therapeutic L1/E7 and L1/L2/E7 chimaera candidate vaccines demonstrated low expression yields and require further optimisation. Transient expression of three HPV-16 L1/L2 chimaeras containing the L2 aa 108-120, 56-81 and 17-36 epitopes in the h4 position in L1 accumulated to high levels in the chloroplasts and were chosen for further analysis in mouse immunogenicity studies.

Plant-derived HPV-16 L1 and the L1/L2 chimaeras assembled into higher-order structures, with distinctive differences in assembly for the L1/L2 chimaeras. The L1/L2(108-120), L1/L2(17-36) and L1/L2(56-81) chimaeras, with 13, 20 and 26 residue sequence replacements, assembled into small cVLPs, capsomere aggregates and capsomeres respectively. As a result, the insertion and the length of the L2 sequence replacement affects assembly and should be considered in the future design of chimaeras.

Mice immunised with partially-purified L1/L2 antigens demonstrated that L1 VLPS and L1/L2(108-120) cVLPs elicited higher anti-L1 responses in comparison to the other chimaeras assembled into capsomeres and aggregates. Overall, L1/L2(108-120) chimaera is the best candidate vaccine, eliciting anti-L1 and L2 responses and neutralising HPV-16 and 52.

Future work includes the optimization of L1/E7 and L1/L2/E7 chimaera expression in plants, possibly by combining the two high-yielding strategies and fusing chloroplast signal sequences to transgenes expressed using a self-replicative vector. In addition, the stability and expression of L1/L2(108-120) in the transgenic lines should be monitored over several generations to determine if the expression system remains commercially viable. Further analysis of L1/L2 chimaera cross-protection remains a priority; the HPV-52 neutralisation assay should be repeated and future assays should include HPV-31 and 35 (which is of particular concern in Africa and South Africa), and HPV-33 and 58 (which are highly prevalent worldwide). Finally, the L1/L2 chimaeras should be re-designed to allow cVLP assembly and improved L2 peptide display. Assembly into cVLPs may enhance both the L1 and L2 immunity and stronger L2 responses may improve the cross-protection of the L1/L2 chimaera vaccines.

Appendix A

Table A1: Plant-expressed HPV-16 L1 antigens and HPV fusion proteins containing HPV-16 L2 and E7

HPV antigen	Plant/tissue	Expression system*	Maximum yields**		Gene modifications	Structural assembly	Reference
			%TSP	mg/kg plant tissue			
HPV-16 L1	Tobacco/leaf, Potato/tuber	Transgenic	0.5% (tobacco) 0.2% (potato)	12 (potato)	Human & plant codon-optimisation, TMV-derived translational enhancer Ω	Capsomeres, VLPs	Biemelt *et al*., 2003
	Tobacco/leaf	Transgenic	< 0.01%	< 0.01	C-terminal truncation (NLS removed)	Capsomeres, VLPs***	Varsani *et al*., 2003b
	Tobacco/leaf	Transgenic	0.08%	-	None	VLPs	Liu *et al*., 2005
	Tobacco/leaf	Transient[a]	< 0.01%	0.04	None	Capsomeres, VLPs	Varsani *et al*., 2006a
	Tobacco/leaf	Transgenic & transient[b]	11 - 17%	530 – 890	Human & plant codon-optimisation, ER & chloroplast localization	Capsomeres VLPs***	Maclean *et al*., 2007
	Tobacco/leaf	Transient[c]	-	550	Human codon-optimisation	Not examined	Regnard *et al*., 2010
	Tobacco/ chloroplasts	Transplastomic	1.5%	-	Plant-codon optimisation, 5' UTR & N-terminal plastid gene sequence (downstream box)	VLPs	Lenzi *et al*., 2008
	Tobacco/ chloroplasts	Transplastomic	20 - 26%	2100 – 3700	Used the light-regulated psbA 5'-UTR	VLPs	Fernández-San Millán *et al*., 2008
	Tobacco/ chloroplasts	Transplastomic	1.5%	-	Mutated with Cys replaced by Ser residues at aa 175 and 428	Capsomeres	Waheed *et al*., 2011
HPV-16 L1/E6/E7	Tomato/fruit	Transgenic	0.1%	-	C-terminal truncation (NLS removed), Fusion of E6/E7 epitopes, ER-localisation sequence	cVLP	Paz de la Rosa *et al*., 2009

[a] Transient expression via tobacco mosaic virus (TMV) or potato virus X (PVX) viral vectors
[b] Agrobacterium-mediated transient expression
[c] Transient expression via agroinfiltration-delivered replicative Bean yellow dwarf virus (BeYDV)-based vector
** Maximum yield expressed as a percentage of the total soluble protein (TSP) and/or expressed as mg of antigen per kg of plant tissue (mg/kg)
*** Mixed population of VLP sizes (within 20-65 nm)

Appendix B

Table B1: Pilot experiments to optimize the purification of plant-expressed HPV-16 L1 and L1-based vaccine antigens

Strategy	Method	Details	Results	Reference
Extraction	*Crude protein extraction	Mechanical methods, sonication, filtration	Antigen soluble and localised in the supernatant.	Chapter 3, Section 3.2.1
Size-based purification	Microfiltration	Cross-flow microfiltration cartridges (0.45 & 0.65um)	Process timely. Low antigen recovery: although the majority of the antigen was detected in the permeate, antigens were retained, even using the larger 0.65um cartridge.	The technical extract from "The Innovation Fund Final Report: Novel Papillomavirus Vaccines, January 2007" (Rybicki, 2007; unpublished), and Cook *et al*. (1999)
	Ultracentrifugation	40% sucrose cushion + 30% CsCl gradient	No distinct bands visible, protein broadly detected in several fractions & method not reproducible for different L1 chimaeras.	Varsani *et* al. (2003a & b); Fernández-San Millán *et al*. (2008); Schellenbacher *et al*. (2009)
		5-30% continuous sucrose gradient + 30% CsCl gradient	No distinct bands visible in either gradient & protein broadly detected in several fractions. Large loss in recombinant protein yields.	Based on method 5B, except a continuous 5-30% gradient was used.
Chromatography purification	Cation-exchange chromatography	POROS 50HS column	Majority of antigen did not bind column. A small proportion of antigen bound strongly and irreversibly to the column	As per manufacturer's instructions. Described by Cook *et al.* (1999) to purify yeast-expressed HPV-11 L1
		HiTrap SPFF column	Majority of antigen did not bind column.	As per manufacturer's instructions
	Heparin chromatography	*HiTrap Heparin HP column	All chimaeras and L1 bound the column and eluted with a high salt PBS.	As per manufacturer's instructions. Described by Johnson et al. (2009) and Kim et al. (2009, 2010) for the purification of L1 from HPV-5, 16, 18, and 31.
Concentration/ buffer exchange	Ultrafiltration	Cross-flow ultrafiltration cartridge (300 kDa MWCO)	Process timely. Degradation detected. Antigen successfully concentrated.	The Innovation Fund Final Report: Novel Papillomavirus Vaccines, January 2007 (Rybicki, 2007; unpublished)
		*Macrosep® ultafiltration spin tubes (10 kDa MWCO)	Rapid concentration and desalting of chromatography fractions	As per manufacturer's instructions

* Methods selected for the final purification of vaccine antigens

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